DEVELOPMENT OF MODIFIED TEMPORARY IMMERSION TECHNOLOGY FOR MASS PROPAGATION OF SOME VALUABLE PLANTS

BOWORN KUNAKHONNURUK

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in (Biotechnology)
2018

DEVELOPMENT OF MODIFIED TEMPORARY IMMERSION TECHNOLOGY FOR MASS PROPAGATION OF SOME VALUABLE PLANTS

BOWORN KUNAKHONNURUK

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in (Biotechnology)
2018

Thesis entitled "Development of modified temporary immersion technology for mass propagation of some valuable plants"

By BOWORN KUNAKHONNURUK

has been approved by the Graduate School as partial fulfillment of the requirements

for the Doctor of Philosophy in Biotechnology of Naresuan University

Oral Defense Committee

..... Chair
(Associate Professor Dr. Prasart Kermanee)

..... Advisor
(Assistant Professor Dr. Anupan Kongbangkerd)

..... Co Advisor
(Dr. Phithak Inthima)

..... Co Advisor
(Assistant Professor Dr. Somjit Homchan)

..... Internal Examiner
(Associate Professor Dr. Duangporn Premjet)

Approved

.....
(Professor Paisarn Muneesawang)

for Dean of the Graduate School

Title	DEVELOPMENT OF MODIFIED TEMPORARY IMMERSION TECHNOLOGY FOR MASS PROPAGATION OF SOME VALUABLE PLANTS
Author	BOWORN KUNAKHONNURUK
Advisor	Assistant Professor Dr. Anupan Kongbangkerd
Co-Advisor	Dr. Phithak Inthima , Assistant Professor Dr. Somjit Homchan
Academic Paper	Thesis Ph.D. in Biotechnology, Naresuan University, 2018
Keywords	Temporary immersion system, Mass propagation, Conservation, Secondary metabolite, Epipactis flava, Calanthe rubens, Drosera communis, Bacopa monnieri

ABSTRACT

Temporary immersion system, one of the effective culture systems in plant tissue culture technology has been successfully used in different purpose. Based on a principle of plant bioreactor, the general operation of this system allowed intermittent contact between the explants and liquid media. Although, various designs of TIS have been distributed on market. Amount of them are still expensive and non-flexible modification to use. Therefore, the completely twin-bottles TIS was successfully established in the final development version for further comfortable and flexible utilization. So, the efficient function of versatile TIS had been accurately investigated by comparative culture systems for mass propagation of some valuable plant species. Regarding with plant conservation, *Epipactis flava* as an endangered rheophytic orchid of Thailand was successful for large scale multiplication using TIS in term of a number of new shoots, shoot buds, shoot height and leaf per explant. After acclimatization, the highest survival percentage of plantlet was observed in TIS. Furthermore, 76.7 and 60% of plantlets could survive after 8 weeks of transplantation to cultivate in an artificial stream. For comparative systems of *Calanthe rubens* cultivation, the significant highest plant survival rate could obtain from SSS and TIS, whereas the good growth parameter including the highest number of new shoots, roots, leaf and pseudo-bulblet diameter could obtain from CIS. However, in

transplantation step, the highest percentage of survival was found in plantlets derived from SSS. In case of *Drosera communis*, TIS was offered the most effective system for mass production. Growth response of *D. communis* explants was significantly influenced by TIS more than conventional culture techniques. The highest number of proliferated shoots and biomass production, as well as yield of plumbagin, was displayed in TIS. For the cultivation of *Bacopa monnieri*, using TIS with different immersion time of frequency and duration, the results revealed that 3 times per day of medium feeding at 10 min per time was the best immersion condition for promoting growth, biomass and bacoside yield production of *B. monnieri*. Furthermore, 20 explants of *B. monnieri* per vessel offered the most suitable density of inoculum for large-scale biomass and bacoside yield production. In order to investigated propagation efficiency of *B. monnieri*. The different culture systems including TIS, CIS and SSS were performed. The highest of shoot numbers per explant, shoot height, node number per shoot and biomass production was exhibited in TIS. Although, bacoside content was found in SSS and CIS higher than TIS, significantly. But the highest of bacoside yield was obviously observed from TIS. So, the development of simplified temporary immersion system in this research provided flexible operation, easy to handle and convenient modification for further exploration.

ACKNOWLEDGEMENTS

First of all, I would like to express thankfulness to the members of my thesis committee. Assistant Professor Dr. Anupan Kongbangkerd, you are a wonderful advisor and helping me in everything and every problem that come into my research throughout the period of time. Thanks for kindliness and accepting me into this plant tissue culture research unit. I gratefully thank my co-advisor, Dr. Phithak Inthima, that you sacrifice your precious time to take care of me and to provide the best advice for this research. Great thank, Assistant Professor Dr. Somjit Homchan for providing knowledge about the research procedure.

I would like to thank everyone for the support and helping of my work. Furthermore, my special gratitude is given to my parents, who are everything in my life.

Thank you to Plant tissue culture research unit, Department of Biology, Faculty of science, Naresuan university for providing tool and facility of this research. Great thank, Science Achievement Scholarship of Thailand for providing scholarship since master's degree to doctor's degree.

BOWORN KUNAKHONNURUK

TABLE OF CONTENTS

Page

TABLE OF TABLES

Page

TABLE OF FIGURES

Page

CHAPTER I

Introduction

Background

Tissue culture is the in vitro culture of cells, tissues, organs or whole plants on artificial culture medium under controlled sterile conditions and environment. Under in vitro conditions, growth and development of cultured tissues are controlled by medium components especially plant growth regulators and environmental factors i.e. gaseous, light and temperature etc. In the past, plant tissue culture was exploited as a basic research tool for rapid propagation, disease-free plant production, plant improvement, in vitro preservation as ex situ conservation and secondary metabolite production. Nowadays, the system of cultivation has been rapidly developed and improved to increase plant production potential for several requirements. Generally, the micropropagation by plant tissue culture techniques has been done by cultured the plant in semi-solid or liquid culture medium using small containers. However, it is laborious, high production cost and high risk of contamination rate that are required a number of carefully handling steps during multiplication process and also the large number of culture vessels and culturing area are needed. Therefore, these conventional culture are unfavorable for large-scale micropropagation. In order to decrease the limitations, efficient culture system has been continuously developed the bioreactor system has also been developed using a large container for mass production. However, it is not suitable for plant development or differentiation due to appearing shear stress from impellers and continuous immersion into liquid medium and caused physiological problems such as asphyxia, hyperhydricity or abnormal development. Hence, the efficient culture system for plant mass production has been created to eliminate their limitations. Temporary immersion system (TIS), an advanced technique for efficient in vitro mass propagation, has been recently developed base on plant bioreactor technology to enhance the propagation efficiency using liquid system. The effective of this system is combined advantages of both

semi-solid and liquid systems. Several studies are now successfully reported to use TI system for in vitro propagation and conservation of ornamental and medicinal plants.

Presently, various types of TIS have been individually invented or commercially launched such as RITA®, SETIS® and PLANTIMA®. Although, various types of TIS have been sold in the markets, their constructions of culture volume are limited not only the height of growing headspace in culture vessel, high risk of contamination in some TIS form and hard to apply with a wide range of plant species, but also there are expensive due to most of them need to import from abroad. Therefore, the development of versatile in-house TIS was necessary. In this study, a simple installation, economically cost and versatile TIS was developed. The efficiency of TIS in the term of mass propagation and biomass production of some valuable plants were performed and compared with conventional semi-solid and liquid culture system.

Objective

To develop temporary immersion system for mass propagation of some valuable plant species

Scope of research

In this study, twin-bottles temporary immersion system was developed. The mass propagation efficiency of TIS was compared with semi-solid, continuous immersion and temporary immersion culture systems. Survival and ex vitro growth of plantlet obtained from different culture systems was also studied and compared. Moreover, some factors affecting plant growth and development in TIS were examined to obtain the suitable operating condition of temporary immersion system.

Keywords

Temporary immersion system, Mass propagation, Conservation, Secondary metabolite, *Epipactis flava*, *Calanthe rubens*, *Drosera communis*, *Bacopa monnieri*

Expected benefit gains

1. To know the appropriate culture condition of temporary immersion system for improving mass propagation and/or secondary metabolite production of some valuable plants.

2. To construct a prototypic multipurpose temporary immersion system for mass propagation and/or secondary metabolite production of some valuable plants.

CHAPTER II

Establishment of Temporary Immersion System for In Vitro Plant Culture

Summary

Temporary immersion system, as tools in plant tissue culture technique has been proposed of micropropagation, based on a principle similar to plant bioreactor, preferring temporary contact between the explants and liquid culture medium more than permanent contact. Although various designs of TIS have been distributed on markets in last few years, but, amount of available TIS are still expensive and non-flexible to use. Therefore, the establishment of versatile in-house TIS was necessary. After development, the complete twin-bottles TIS was represented in the final version, that consisted with effective culture container system, flexible automated controller and also easy adjusted environments like the light or air flow rate. So, the establishment of temporary immersion system for in vitro plant culture could be enhanced the efficient application.

Introduction

Plant bioreactor and temporary immersion system

Plant tissue culture has been revealed to be a highly effective method for large number of plant production with minimum usage of space. Its success to use and apply for clonal micropropagation of commercial crops, improvement of plant variation, conservation of endangered plants as well as enhancing the production of essential bioactive compounds. Apart from their use as a method of plant researches in all aspects. The past decades of plant tissue culture technology has evolved to simplify micropropagation and sustain continuous production. A number of steps and numerous approaches have been developed. Especially technology of bioreactors, they are the most prominent being adapted and served many advantages of mass production (Akin-Idowu, Ibitoye, & Ademoyegun, 2009; Debnath, 2011). Several bioreactors are available providing the optimum culture condition by regulated

various nutrition and physical factors for efficient productivity (Afreen, 2006; K. Y. Paek, Chakrabarty, & Hahn, 2005; Yesil-Celiktas, Gurel, & Vardar-Sukan, 2010). Normally, utilization of bioreactors requires the use of liquid instead of agar gelled media during proliferation and biomass accumulation stages. Hence, production costs and handling of medium preparation are improved (R. V. Sreedhar, Venkatachalam, & Neelwarne, 2009; Ziv, 2005). Furthermore, liquid medium allow the close contact of explants with the medium, which may facilitate and stimulate the uptake of nutrients and growth regulators leading to rapid growth and development of plant material (Mehrotra, Goel, Kukreja, & Mishra, 2007). In addition, it's easy to renew the culture medium without transfer to new culture container (Berthouly & Etienne, 2005; Sandal, Bhattacharya, & Ahuja, 2001). In term of large-scale micropropagation, bioreactors have been industrialized as alternative way providing mass production. Nevertheless, their applications is often limited and is not always easy to utilize. Frequently, most of explants cultured with bioreactors are completely submerged in liquid medium throughout period of cultivation. Therefore, various problems, asphyxia or abnormal development can arise when prolong continuous immersion similar to liquid culture system (Ziv, 1999). Indeed, bioreactor system is originally designed for microbial cultures, so it may not suitable for plant differentiation or regeneration. This is because of high shear force creating by aeration system which not specifically desired for plant cells, tissues, organs or whole plant cultures (Berthouly & Etienne, 2005; Georgiev, Schumann, Pavlov, & Bley, 2014; Teisson & Alvard, 1999). In order to secure the plant growth, development and physiological integrity, those bioreactors for plant cultivation need to be developed. Establishment of plant bioreactors could be classified into several types depend on working methods (Debnath, 2011). Temporary immersion system (TIS) is a new generated technology of plant bioreactors. This system do not use mechanical agitation devices, but relying on dislocation of liquid medium by hydrodynamic forces during immersion period. Thus, under these conditions, some limitations occurring in former bioreactors was eliminated and as well improve the morphology and physiology of propagating plants (Etienne & Berthouly, 2002; Teisson & Alvard, 1999). However, this system was still not always easy to use, but currently worldwide in plant tissue culture technology. After many years of research and development. Until now, various features of TIS

have been created and functioned, see in Georgiev et al. (2014). Their systems have been successfully applied for mass propagation of important plants (Ducos, Terrier, Courtois, & Pétiard, 2008; Mosqueda Frómeta, Escalona Morgado, Teixeira da Silva, Pina Morgado, & Daquinta Gradaille, 2017; Sharma, Gupta, Khajuria, Mallubhotla, & Ahuja, 2015; Watt, 2012).

Amount of available TIS that mention before, Twin-flasks or -bottles temporary immersion system, more simple and flexible construction, has popular in several commercial plants. Additionally, this system is easier to set up and less expensive than the other TIS designs. Thus, twin-bottles TIS has been frequently modified and created using cheaper material or equipment for establishment by own researchers (Godoy et al., 2017; Moreira, Silva, Santos, Reis, & Landgraf, 2013; Mosqueda Frómeta et al., 2017; Topoonyanont, Pumisutapon, Klayraung, & Poonnoy, 2017). Currently, many designs and conformations of TIS have been distributed on the market in the last few years, However, they are still expensive because most of them need to import from abroad. Moreover, the construction of their systems are not flexible and no easy to handle due to sophisticated equipment. In order to fully utilized, the development and establishment of versatile in-house TIS was necessary. Therefore, the objective of this research was to develop twin-bottle temporary immersion system and ascertain whether an establishment TIS had been benefited to further study.

Materials and Methods

The establishment of twin-bottles temporary immersion system was initially carried out in Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok. Which all equipment and tools were attempted to obtain the possible cheapest materials along with more effective for TIS production.

General description of twin-bottles temporary immersion system

Generally, three main operational systems are involved with the installation of twin-bottles TIS. 1) Container system; twin-flasks TIS consist of two vessels, one is a plant chamber (culture vessel), whereas another one is medium reservoir (medium

vessel). Each vessel is connected to pressured air tube lines and coupled with solenoid valves, the air directional controller. Before entering into culture vessels, the air is sterilized by membrane air filter, linked with the silicone tube on top of each vessel. The plant chamber may or may not be contained with support material for trig explants such as glass beads, polyurethane foam, metal or nylon sieves. 2) Pneumatic system; a source of compressed air released to impel liquid culture medium in both vessels, which air pressure is controlled by regulating valve. 3) Automatic controller system; using a computerized, that designed to monitor and control cultivation conditions (**Figure 2.1**). In term of working procedures of twin-bottles TIS, four steps of operation are followed through including stationary phase (A), no compressed air is supplied, liquid medium is retained in the reservoir vessel; immersion phase (B), the compressed air is supplied into reservoir vessel continuously in order to drive liquid medium to culture vessel, the cultured plant is immerged in liquid medium and uptake nutrients for few minutes and constantly continued to provide oxygen for plant during immersion period; end of immersion phase (C), redirection of air compression begin to transfer liquid medium back to medium vessel; ventilation phase (D), compressed air is still supplied into the plant vessel for a moment after medium return to the medium vessel in order to renew internal gas environment (**Figure 2.2**).

First version of TIS development

First prototype of TIS development was required to construct following description that the culture container was utilized with two vessels with 0.7 liter glass jars (TG757 jars), inexpensive vessel and resistant to autoclaving. Inside of growth vessel doesn't placed supporting materials (**Figure 2.3a**). The lids of these containers were made two holes and equipped with brass hose fittings (3/8 inch male and female NPT x 3/8 inch hose barb), that they could be purchased from general agricultural or constructional equipment stores (**Figure 2.3b-d**). Both vessels were connected together with silicone tube (Ø 8 mm) and another one tube was attached to membrane filter (0.2 µm PTFE air filter; Acro®50) (**Figure 2.4a**). Whereas, twenty-seven sets of completed TIS was the maximum installation on three layers chromium shelve (**Figure 2.4c, e**). However, this version of TIS was not included automatic controller system and the maximum setting of immersion interval could be defined only three

programs. For light system, fluorescent lamps were fixed to use for plant growth and they were controlled by analog timer. The pneumatic system, a traditional air compressor, source of compressed air was utilized to push the liquid medium. Whereas, the air flow rate could not individually regulate in each TIS vessel.

Second version of TIS development

In this version, their culture containers of TIS from previous version were remodeled to be a larger vessel using 1 liter glass bottle (Ø 80 cm wide mouth; DURAN®). The inside of growth vessel placed with small glass bead (Ø 0.3 cm) to support plant materials (**Figure 2.3e**). Subsequently, tube connectors attached to the caps of bottle were readjusted to be a smaller tube (1/8 inch male and female NPT x 1/8 inch hose barb) and in order to ensure the minimal leakage, O-ring rubber sheets were supplemented into those lids (**Figure 2.3f-h**). Both vessels were linked together with silicone tube (Ø 5 mm) and the end of one silicone tube was covered with stainless fine net to prevent glass bead passed in through tube. Another side of connector tube was attached with membrane filter like as mention before (**Figure 2.4b**). Whereas, twenty-seven sets of completed TIS was the maximum installation in three layers chromium shelve (**Figure 2.4d, f**). Furthermore, in this version, TIS had complemented with automatic controller system to regulate sequence of process, controlling the electric current for turn-on/off of twelve-solenoid valves. Whereas, the programmable timer was determined by software of Arduino IDE written by researcher. Therefore, the automatized immersion interval of TIS could take in three functions. Light system included within system controller was used with the LED lamps for plant cultivation. For pneumatic system, air compression obtained from oil free air pump was supplied for dislocation of liquid medium. However, limitation of air flow rate reached to culture container could not be adjustable in each TIS vessel.

Final version of TIS development

The establishment of twin-bottles TIS was approached to final development. In this version, the culture containers feature still look like the second version (**Figure 2.3i**), but difference in few devices. That the tube connectors were newly designed to be seamless connector tubes and as well coated with thin chromium. In particulars,

two different types of tube were composed with one piece, it was designed as a hose barbed bulkhead fitting (Ø 1/8 inch hose barb) used for connection between culture containers and another piece, it was molded only one side of hose barbed fitting (Ø 1/8 inch hose barb) (**Figure 2.3j**). Which both tubing connectors were installed at the lids of culture containers and also added with O-ring rubber sheets like as above mention (**Figure 2.3k-l**). Silicone tube and membrane filter were used as the same second version. Which the complete components of culture system of final version could see in **Figure 2.5**. Moreover, thirty complete sets of twin-bottle TIS could be installed for the maximum application on three layers chromium shelve (**Figure 2.6a**). Besides, automatic controller, the programable timer to control the electric current into the twenty-four solenoid valves, was augmented. Apart from working potential of previous systems, in this version, TIS could be separated operation up to six programs within one shelve. In addition, the individually appropriate adjustment of air flow rate could be possible due to the air valve was coupled within each culture container (**Figure 2.6b**). Light system was also included within system controller and LED lamps were easy to modify for various culture condition (**Figure 2.6c**). For pneumatic system using oil free air compression like previous TIS version.

Results

Establishment of twin-bottle temporary immersion system, culture container system, automatic controller system and pneumatic systems had been successfully developed to supply facilitation and increase ability for further use. Base on principles of twin-bottle TIS, the procedures commonly involve air pressure, thus various joints of structure must be concerned and should be improved. Particularly, a part of culture container and tube connector, that they are the main important construct of using. Detailed descriptions of all materials required and the practice for constructing the equipment is descripted in the material and method section. The performance and limitation of twin-bottles TIS were investigated after initial working.

First version of developing TIS, culture system had been used by glass bottles attached with brass tube for connecting silicone tube on the lids, which this design is cheap of culture container and does not need sophisticated equipment. However, after TIS operation, the major limitation of this version was found that operation of TIS

was inconvenient because, this version didn't have the controller system for automatized immersion. So, the manual procedure for working would be done by researcher. Moreover, during immersion and ventilation, air pressure or liquid medium had been highly leaked at lids and connector tube, that led to risk of culture medium contamination. Whereas, air compression obtained from a traditional air pump was used in this system. Which the entrained oil generated form motorized rotor might be mixed with air flow and interfered with proper function of TIS, as like source of contamination.

From these restrictions and deficiencies, the twin-bottles TIS in second version has been upgraded for more effectiveness from previous models. Duran bottles were replaced to use as culture container, apart from being readily available, autoclavable, long time for usage and it was easily modified as well as the price was moderate. Moreover, connecting tubes were altered use to be a smaller brass tubes. Therefore, size of air bubble occurred within growth vessel could easier be adjusted. But it still difficult to control to be a similar in every bottles. While, after autoclaving about three or four times, the connector tubes made from brass materials were frequent a corrosion. That might be effect on culture medium efficiency. Furthermore, this TIS version was improved the leakage of air pressure or liquid medium by adding O-ring rubber sheets into the lids of culture containers. In term of automatically operation system of TIS, Arduino, electronic platform based no easy to use does not require complex hardware and software, was applied to control the sequence of operations in automatic control system. Thus, the comfortably functional operation of TIS had been optimized. In order to improve pneumatic system, the cleansing air passed into culture container was originated from oil-free air pump for reduction of contamination as much as possible. However, these results of twin-bottles TIS development of second version were not yet satisfied as it should be. Accordingly, the establishment of TIS should be developed again.

In an attempt to accomplish a further optimization of twin-bottles TIS, in the final version, key improvement of this version was targeted to the connector tubes, that they were designed to be a seamless tube. Besides, those metal tubes were coated with thin chromium to protect oxidation after autoclaving, that occurred in brass materials composed in first and second TIS version. Which oxidizing appearance, it

might be interfered or contaminated into liquid culture medium. So, the leakage of air or liquid medium and also tube oxidization were almost disappeared. Furthermore, installation of the ball valves within the individual channel of culture container allow easier to regulate the air flow rate passed into each culture container than previous versions. Regard of useful space of shelves, this version could provide maximum utilization containing up to thirty culture containers. Due to the tubing line and electric-solenoid valve position were rearranged. Consequently, the working potentiality of this version could be determined up to six difference of immersion intervals in ordered requirement. In summary, the comparative properties of three different versions of complete set twin-bottles temporary immersion system were presented in **Table 2.1**. Comparative complete set and commercial TIS of general features, advantages and limitations was offered in **Table 2.2**.

Discussion

Various approaches could be used to success the plant mass production in plant tissue culture, not only using conventional techniques with small culture container but also using innovative cultivation methods for large scale production. Since 1983, temporary immersion technology has been developed and tested to become the most effective culture system for plant cultivation (Berthouly & Etienne, 2005; Georgiev et al., 2014; Watt, 2012). All these systems respect the conditions mentioned by Teisson and Alvard (1999); avoid consistently continuous immersion into liquid medium.

Recently, numerous published reports revealed that many researchers have achieved the development and establishment of their systems with superior application. The appearance of TIS differ in design and size of containers (An, Kim, Moon, & Yi, 2016; Ducos, Labbe, Lambot, & Pétiard, 2007; Godoy et al., 2017; Kokotkiewicz, Bucinski, & Luczkiewicz, 2015; Welander, Persson, Asp, & Zhu, 2014; Zhao, Sun, Wang, Saxena, & Liu, 2012), utilization of computerized system or simple timers for immersion determination (Ahmadian, Babaei, Shokri, & Hessami, 2017; Lyam, Musa, Jamaleddine, Okere, & Odofin, 2012; B. Zhang et al., 2018), through use a peristatic pump or air compressor or mechanical motion of container to displace liquid medium (Moreira et al., 2013; Mosqueda Frómeta et al., 2017;

Welander et al., 2014). Which, the most of them were confirmed the production efficiency by many researchers. The results of this study had been achieved to improve new automated operation and configuration based on principle of TIS for further comfortable and flexible utilization. Thereby, the challenges of this establishment were, how to decrease the limitations or deficiency presented from previous version of TIS development, along with increase the optimization to new version. Which, the completely final version of establishment of TIS was represented in this chapter to use for further experimentation.

Conclusion

Improvement of new operation and construction of twin-bottles temporary immersion system has been successful establishment in the final version. That the finalized TIS had been installed various devices, for instance, supplementary novel automatic controller used programmable timer for controlling immersion interval, seamless connector tubes developed for connection culture container, flexible light system was easier to modify for different lighting condition, in order to increase performance and accommodation for further employment.

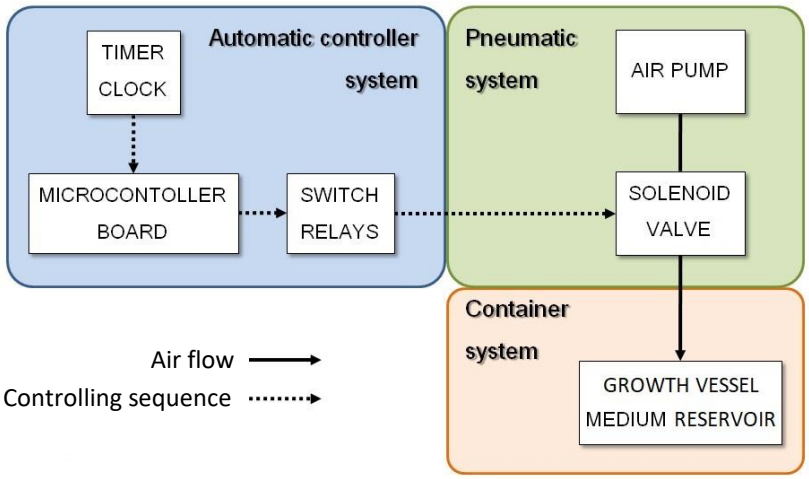

Figure 2.1 Schematic diagram represent the operation of three main systems of temporary immersion system in this study.

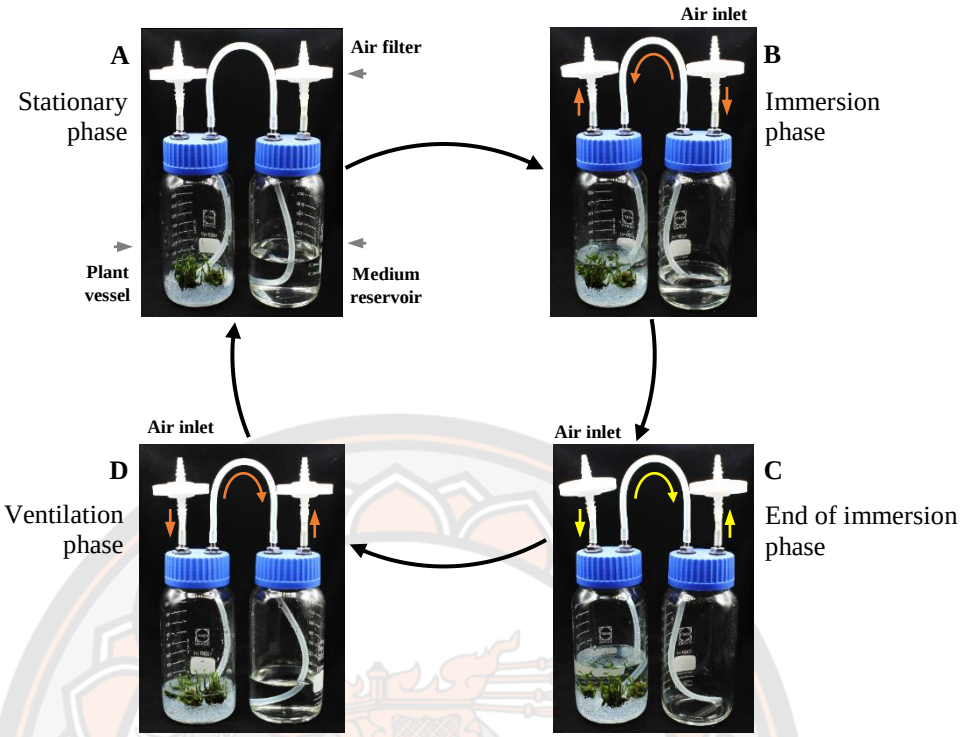

Figure 2.2 The operational cycle of twin-bottles temporary immersion system: stationary phase (A), the culture medium is remained within the medium reservoir, no air is supplied; start of immersion phase (B), the culture medium is dislocated from medium reservoir to plant vessel by air pressure and constantly continued during immersion period; end of immersion phase (C), redirection of air compression begin to reverse liquid medium into medium reservoir; ventilation phase (D), the air is compressed to plant vessel in order to push the medium back to medium reservoir and renew the air inside the culture vessel.

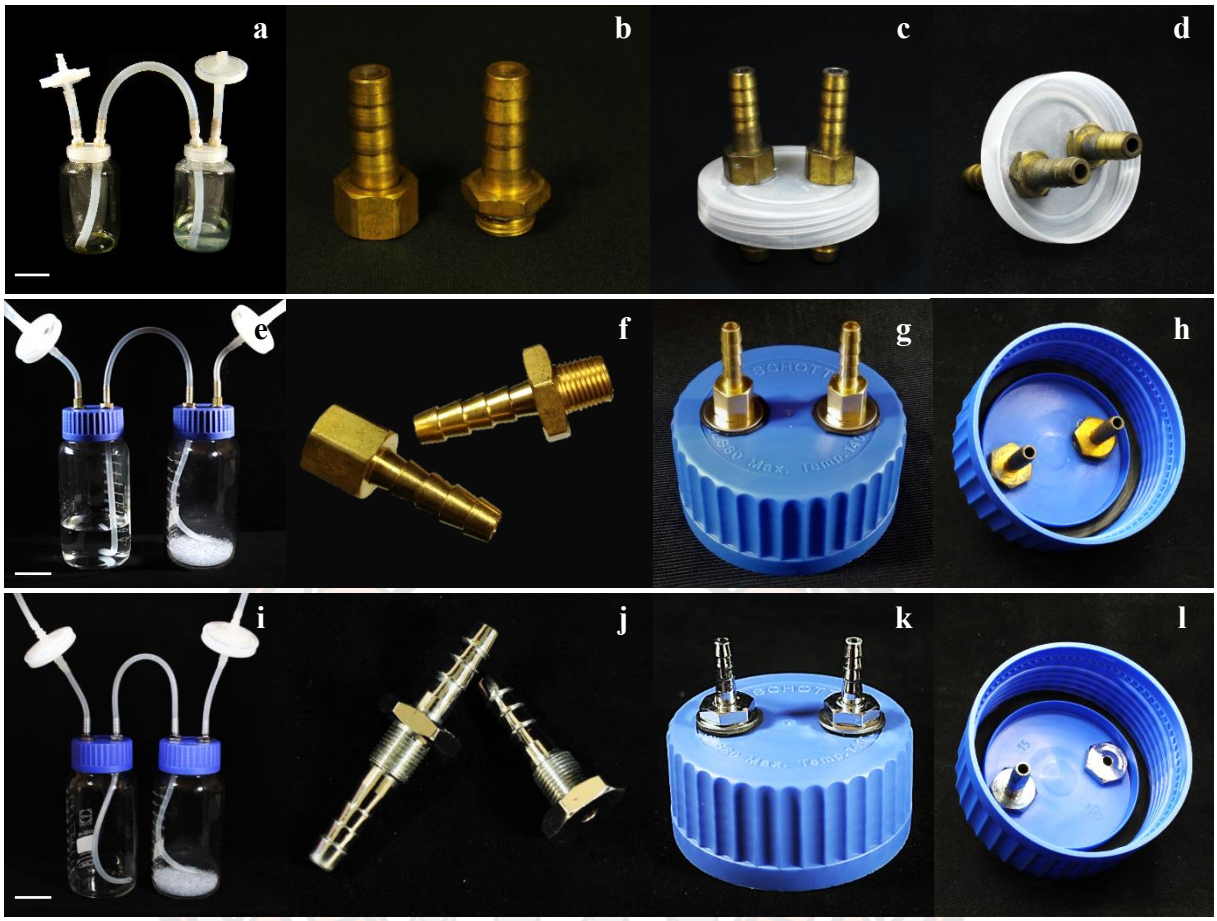

Figure 2.3 Comparison between culture containers (a, e, i), tube connectors (b, f, j) and screw caps with tube connectors (c-d, g-h, k-l) of version I (a-d), II (e-h) and III (i-l) establishments of temporary immersion system in this study.

Figure 2.4 Comparison between the complete sets of the version I (a, c, e) and II (b, d, f) of temporary immersion system.

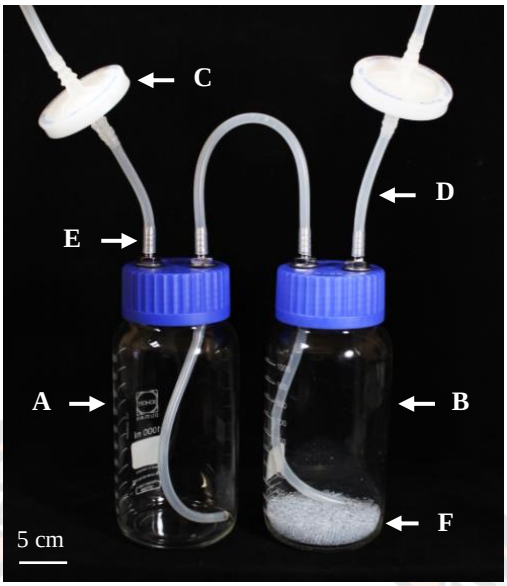

Figure 2.5 The complete components of the final version (version III) of twin-bottles temporary immersion system including 1 L medium reservoir (A), 1 L plant vessel (B), 0.2 μm PTFE air filters (C), 5 mm Ø silicone tube (D), hose tubing connectors (E) and 3 mm Ø glass bead (F).

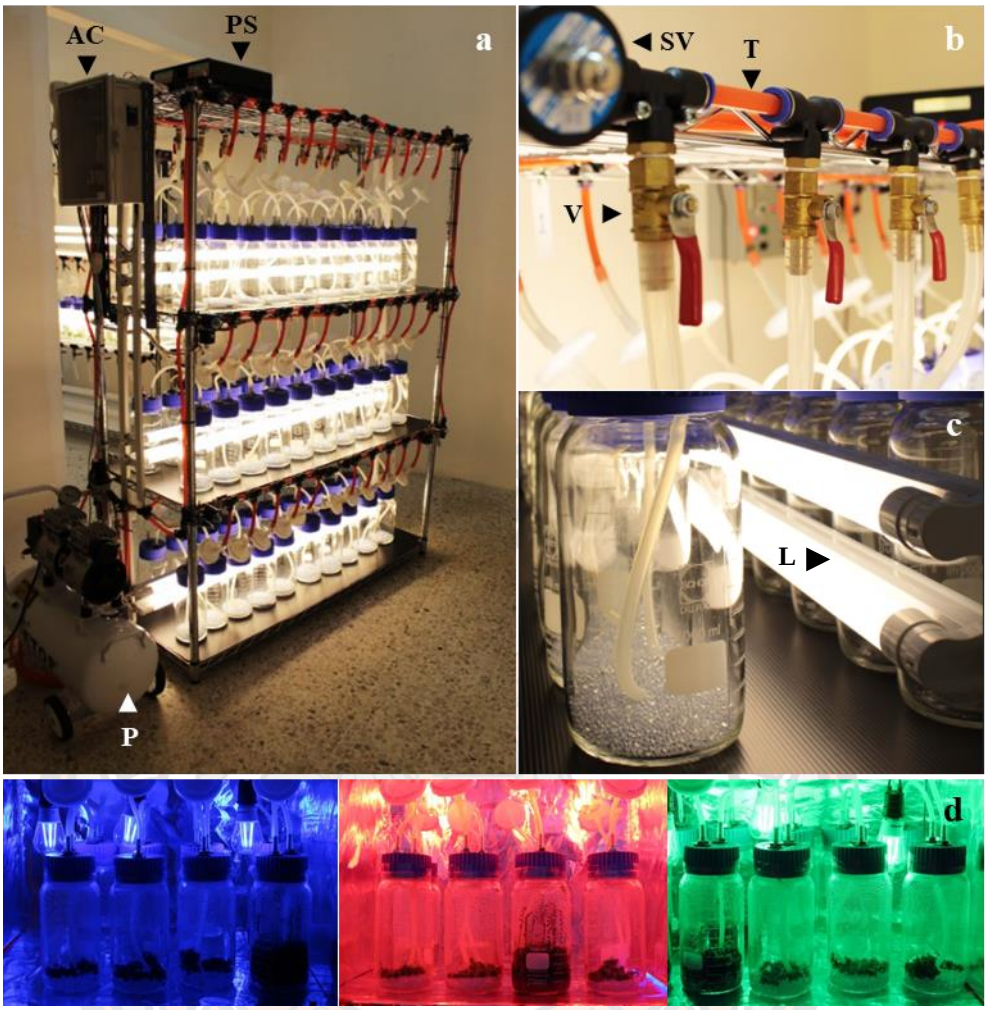

Figure 2.6 The complete set of the final version (version III) of temporary immersion system (a) consisted of oil-free air pump (P), Automatic controller (AC) and power supply (PS). Pattern of gadgets connection (b) including air tube (T), air valve (V) and solenoid valve (SV). Light system (c-d) were normally composited with warm-white LED lamp (L) and able to modify difference of light quality for further culture condition.

Table 2.1 Comparative properties of three versions of complete set temporary immersion system that was developed.

	Version of TIS		
	I	**II**	**III**
System controller			
Type of controller	Manual	Automatic	Automatic
No. of maximum immersion program setting	3	3	6
Minimum immersion time (min)	0.5	1	1
TIS Container			
Types of container	TG757 Glass bottle	Duran®-wide mouth	Duran®-wide mouth
Volume (L)	0.7	1.0	1.0
Surface area (cm^2)	50.2	78.5	78.5
Maximum medium volume (mL)	400	900	900
No. of TIS / set	27	27	30
Type of Tube connector	Male-female brass	Male-female brass	1 piece chrome-plated brass
Seamless connector tube	×	×	✓
Oxide of tube after autoclave	✓	✓	×
Supporting material at the bottom of plant vessel [1]	None	Glass bead	Glass bead
Risk of air or medium leakage [2]	High	Low	None
Pneumatic system			
Air flow rate can be adjusted for each container	×	×	✓
Oil free air compressor	×	✓	✓
Light system			
Type of light	Fluorescent	LED	LED
Lighting time controller	Analog timer	Included in system controller	Included in system controller
Easy to modify lighting [3]	×	×	✓

[1] Avoid the immersion of plant material in the medium that remain after ventilation phase.

[2] Criteria of risk of leakage was observed from the leak of pressurized air or liquid medium between connecting points during immersion and ventilation phase: high, more than 30% of connecting point present air or medium leakage; low, less than 10% of connecting point present air or medium leakage; none, no leakage was found.

[3] Modify lighting such as change the position, change the type of bulb, increase or decrease the intensity of light.

Table 2.2 Comparative of general features, advantages and limitations of different TIS systems.

	TIS			
	Invented TIS	**SETIS**	**RITA**	**PLANTFORM**
Construction material	Glass bottle (Duran)	Polypropylene	Polypropylene	Polycarbonate
Compartments of container	Two pieces	Two pieces	Single piece	Single piece
Adjustable size of container for Scale-up	✓	×	×	×
Size of container	1, 2, 5, 10 L	6 L (Growth vessel) 4 L (Medium vessel)	0.9 L	4 L (18x15x15 cm)
Size of headspace for plant growth	Depend upon size of container	About 10 cm high	Small space	About 10 cm high
Maximum medium volume containing	90% of container size	3 L (Recommend)	0.4 L (Recommend)	0.5 L
Container cost (x30 TIS)	70,938 ฿	88,359 ฿	297,420 ฿	54,100 ฿
Pneumatic system cost (x30 TIS)	21,070 ฿	16,000 ฿ (Excluding air compressor)	Unknown	5,407 ฿ (Excluding rack)
Controller system cost	2,835 ฿	80,428 ฿	Unknown	600 ฿
Immersion programs	≥ 6 immersion programs	Only 4 immersion programs	Unknown	Only 1 immersion programs
Total cost of TIS	94,843 ฿ (Complete set)	184,787 ฿ (Excluding air compressor)	297,420 ฿ (Excluding pneumatic and controller system)	60,007 ฿ (Excluding rack; only one immersion program)
Risk of leakage	No risk	Moderate (Container is placed horizontally and liquid medium contact with the lid)	No risk	Moderate (Large lid, not a screw cap)

* Cash exchange rates (EUR to THB) were compared on March 7, 2019.

** Total cost of TIS does not include costs of installation.

CHAPTER III

Comparative Study of Media Formulations and Culture Systems for Micropropagation of *Epipactis flava*, an Endangered Rheophytic Orchid

Summary

Epipactis flava Seidenf., an endangered rheophytic orchid of Thailand is now decreasing rapidly from natural habitat and urgently need to be conserved using ex situ conservation. Effect of semi-solid and liquid state of five different media including VW, MS, BM, MM and KC on seed germination and protocorm development was investigated. The results found that both semi-solid and liquid VW medium could promote the highest seed germination rate (70.2% and 70.4% respectively). However, the highest definite rate of protocorm developmental stage (54%) was found on semi-solid BM medium. In vitro seedlings of *E. flava* were transferred to culture on both semi-solid and liquid VW and MS medium for 2 months. The results revealed that the higher growth parameter as indicated by shoot number and fresh weight could obtain on liquid MS medium. Effect of different culture system; semi-solid (SSS), liquid (CIS) and temporary immersion system (TIS) on shoot regeneration efficiency was also investigated and compared. The results found that the highest percentage of morphological response could obtain from TIS followed by CIS and SSS, respectively. Growth parameters as indicated by the percentage and number of new shoots, shoot buds, shoot height and number of leaf per explant could receive from TIS significantly higher than from SSS and CIS, respectively. Furthermore, the maximum number of new shoots and shoot buds were reliably obtained from TIS, more than 28-29 times compared with SSS and CIS. After acclimatization, the highest survival percentage of plantlet was observed in TIS. Likewise, plantlets derived from TIS could highest survive after 8 weeks of transplantation to cultivate in artificial stream. Therefore, TIS was the most suitable culture system for in vitro mass propagation of *E. flava* when compared to CIS and SSS culture systems.

Introduction

The Orchidaceae is one of the largest families of flowering plants containing approximately 35,000 species (Cribb, Kell, Dixon, & Barrett, 2003). They are fascinating by the diversity of various plant architectures, floral structures, habitats and lifestyle. Nonetheless, rheophytic lifestyle in Orchidaceae appears to be very rare. Only few rheophytic species were reported including *Agrostophyllum laterale* (Schuiteman, 1997), *Epipactis flava* (Seidenfaden & Pedersen, 2002), *Arundina caespitosa* (Averyanov, 2007), *Spiranthes Sunii* (Boufford & Zhang, 2007), *Bulbophyllum rheophyton* (Vermeulen & Tsukaya, 2011) and *Arundina graminifolia* var. *revoluta* (Yorifuji, Ishikawa, Okada, & Tsukaya, 2015). Particularly, *Epipactis flava* is merely found in Thailand, Laos and Vietnam (Seidenfaden & Pedersen, 2002). It is the only rheophytic orchid found in Thailand and considered as endangered species included in red data list of Thailand (Santisuk, Chayamarit, Pooma, & Suddee, 2006). This orchid is grown in very specific habitat area like limestone waterfall, brook and stream that has limestones. Up to date, only ten natural habitats of *E. flava* were discovered in Tak, Nan and Kanchanaburi province of Thailand (Pedersen, Watthana, & Srimuang, 2013). The special life cycle of this orchid is represented in **Figure 3.1**. During the rainy season, only the rhizomes of *E. flava* are remained under the water stream. Thereafter, the shoots are sprouting along with the decreasing of water level (late October) and vegetative parts are growing throughout the cold season. The flowers are produced and bloomed in February, and then the capsules are ruptured around May. Although seeds of *E. flava* could be produced every year but the young germinated seedlings in natural habitat from our observation are not appeared. Furthermore, the loss of particular habitat due to human activities and natural disaster is greatly impacted the existence of *E. flava*. Therefore, the conservation program of *E. flava* must be concerned. In vitro culture, especially asymbiotic germination and seedling cultivation is one of the promising propagation tools for orchid conservation and has been effective in various threatened and endangered orchid species, such as *Paphiopedilum armeniacum* (Y.-Y. Zhang et al., 2015), *Bulbophyllum nipondhii* (Pakum, Watthana, orn Srimuang, & Kongbangkerd, 2016) and *Paphiopedilum insigne* (Diengdoh, Kumaria, Tandon, & Das, 2017). However, the accomplishments of asymbiotic germination are significantly influenced

by various factors. Particularly, the composition of germination media must be concerned (Shekarriz, Kafi, Deilamy, & Mirmasoumi, 2014). The various culture media have been used to find the suitable culture medium for asymbiotic seed germination of each particular orchid species (Dutra, Kane, Adams, & Richardson, 2009; Muthukrishnan, Kumar, & Rao, 2013). For instance, in *Geodorum densiflorum*, the MS medium could promote highest germination rate compared to the other media (Muthukrishnan et al., 2013). Whereas, KC medium was found to be suitable for germination of *Paphiopedilum* seeds (Long, Niemiera, Cheng, & Long, 2010). In addition, some reports suggested that the medium with low concentration of mineral salt like ½ MS or ¼ MS was applicable for seed germination of some terrestrial orchids (Parthibhan et al., 2012; Zeng et al., 2016). Furthermore, the addition of some undefined organic substances such as coconut water and potato extract into the media have been also enhanced the germination rate of orchid seeds (Pakum et al., 2016; Y. Zhang, Lee, Deng, & Zhao, 2013). Beside the medium types, the pollination feature and degree of seed maturity after pollination also had the influence on seed germination rate (Lee, Lu, Chung, Yeung, & Lee, 2007; Pakum et al., 2016; Suzuki, Moreira, Pescador, & de Melo Ferreira, 2012; Zeng et al., 2012). In some orchid species, the germination rate of self-pollinated seed can be reduced due to inbreeding depression, so the cross-pollinated seed is more preferable (Bellusci, Pellegrino, & Musacchio, 2009). Likewise, immature seed of *Cypripedium macranthos* showed a higher germination rate than mature seed (Y. Zhang et al., 2013), contrary result with research of Shimura and Koda (2004) Although asymbiotic germination protocols of orchid seed have been revealed in many species, there is no report in *E. flava*. Since the factors that influence asymbiotic germination of orchid seem to be species-dependent, some factors that may affect asymbiotic germination and protocorm development of *E. flava* were examined in this study. In term of in vitro mass propagation, conventional cultivation technique using semi-solid medium encounter the problems with labor consuming during subculturing period. In order to overcome these problems, liquid shaking system was then developed and improved. Nowadays, several novel liquid culture systems have been invented and reformed included temporary immersion system (TIS). This technology was recognized as a key step for realization of commercial exploitation of plant tissue cultures for mass propagation

(Sharma et al., 2015). Moreover, temporary immersion system, a novel plant bioreactors based on micropropagation have been used to increase the multiplication rate of culturing plants and successfully applied for commercial plants (Moreira et al., 2013; Mosqueda Frómeta et al., 2017; Snyman, Nkwanyana, & Watt, 2011), medicinal plants (M. H. Ibrahim, Chee Kong, & Mohd Zain, 2017; Vives et al., 2017) and endangered plants (B. Zhang et al., 2018). Therefore, these researches provide a possibility for large-scale cultivation of *E. flava* using this technology. Furthermore, greenhouse acclimatization of in vitro derived plantlets is also investigated in order to establish complete propagation program that can be further apply for reintroduction or ex situ conservation. This study is the report offering the comprehensive propagation protocol for *E. flava*.

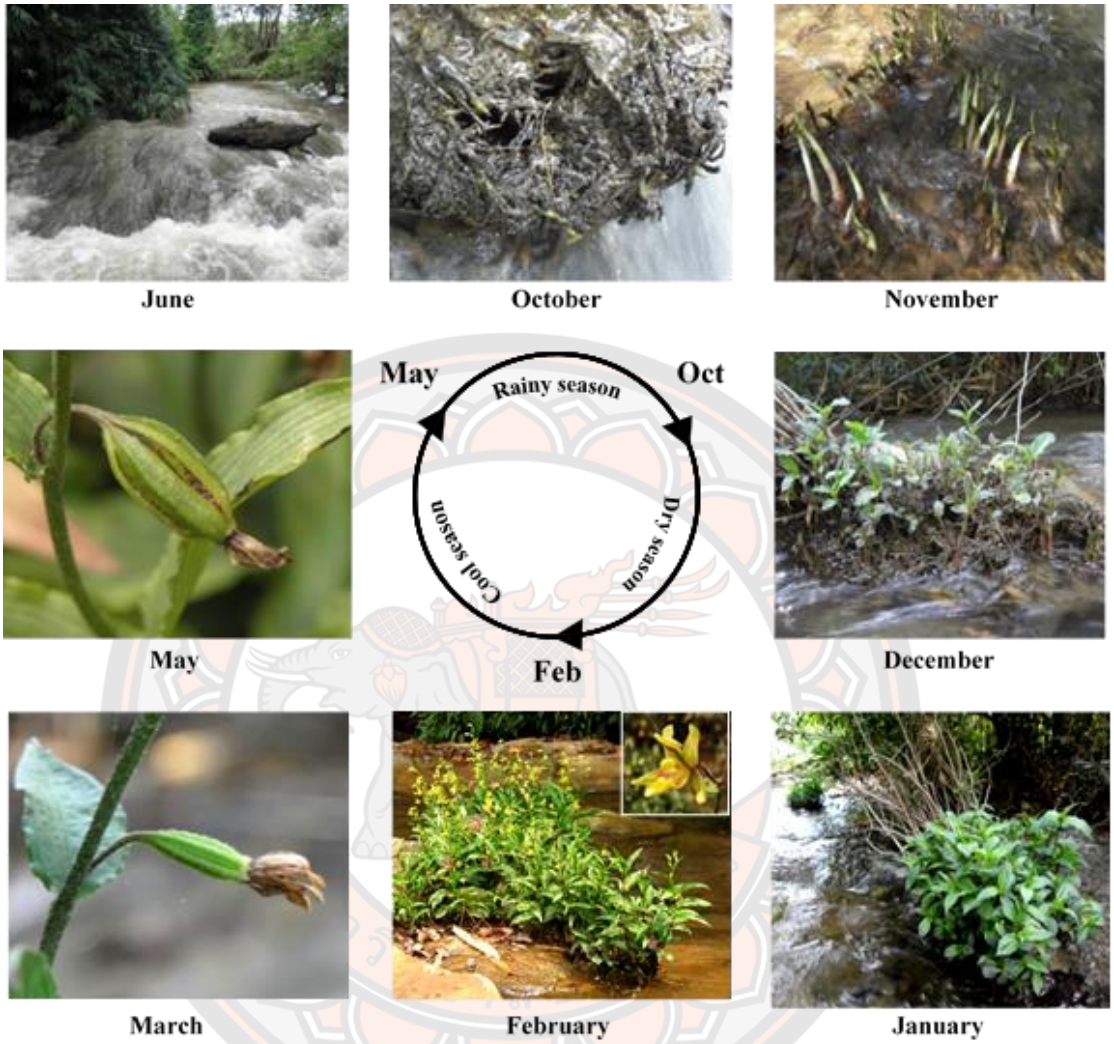

Figure 3.1 Life cycle of *Epipactis flava* in its natural habitat (Huai Tham Suea stream, Mae Sot District, Tak Province, Thailand)

Materials and Methods

Capsule preparation and surface sterilization

Capsules of *Epipactis flava* were obtained from plants growing in natural habitat (Mae-Tao stream, Ban Huai Tam Suea, Mae Sot District, Tak Province, Thailand). In order to prevent the natural pollination, the young inflorescences were firstly covered by fine nylon net bags. When the flowers were opened, the hand cross-pollination were performed, and then the inflorescences were covered again. The capsules were collected at 6 weeks after pollination (WAP) and brought to Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand. For the surface sterilization, the capsules were washed with running tap water for 15 minutes. Thereafter, their capsules were treated with 10% (v/v) Clorox® solution for 10 minutes before rinsed three times with sterile distilled water. Subsequently, the seeds were taken out and mixed together with before further experimentation.

Effect of culture media on asymbiotic germination of *Epipactis flava*

The semi-solid and liquid media of modified Vacin and Went (VW) (Vacin & Went, 1949) supplemented with 150 ml/L young coconut water (CW) and 50 g/L potato extract (PE), Murashige and Skoog medium (MS) (Murashige & Skoog, 1962), BM-1 terrestrial orchid medium (BM) (Purchase from *PhytoTechnology* Laboratories®; Catalog No. B138), Malmgren modified terrestrial orchid medium (MM) (Malmgren, 1996), (Purchase from *PhytoTechnology* Laboratories®; Catalog No. M482) and Knudson C (KC) (Knudson, 1946) were used to investigate the germination efficiency of *E. flava* seed in vitro. All media were added with 20 g/L sucrose. The pH was adjusted as follows: VW 5.2; MS 5.8; BM 5.5; MM 5.75 and KC 5.2. The semi-solid media were solidified with 7.5 g/L agar. The media were autoclaved at 121 ^{o}C for 15 minutes before use. Thereafter, the mixed seeds of *E. flava* were sown on/in their media. The liquid cultures were agitated at 110 rpm with orbital shaker. All seed cultures were maintained at 25±2 ^{o}C under the dark condition. The seed germination and protocorm development of *E. flava* were observed and classified according to criteria from Kunakhonnuruk, et al. (2018) (**Figure 3.3**) under

stereo microscope and recorded every 2 weeks for 8 weeks. Five replications with 100 seeds per replication were inspected for each treatment.

Effect of culture media on in vitro culture of *Epipactis flava* seedling

To established suitable culture medium for in vitro seedling of *E. flava*, VW and MS media in both semi-solid and liquid culture systems were investigated for cultivation of seedlings obtained from previous experiment. All the media were supplemented with 150 ml/L CW, 50 g/L PE and 20 g/L sucrose. For the semi-solid medium, 2 g/L activated charcoal and 7.5 g/L agar were added. The pH was adjusted to 5.2 for VW and 5.8 for MS media. The liquid cultures were shaken at 110 rpm with orbital shaker. Their cultures were incubated under cool-white fluorescent lamp (20 µmol/m^2s light intensity) for 12/12 hours light/dark photoperiod at 25±2 ^oC. After 8 weeks of culture, the growth parameters such as percentage of new shoot formation, number of shoot and shoot height were recorded. All the treatments were consisted of fifteen seedlings.

Comparative study on efficient micropropagation system in *Epipactis flava*

In order to investigate propagation efficiency of *E. flava* in different culture systems, semi-solid system (SSS), continuous immersion system (CIS) and temporary immersion system (TIS) were performed and compared. In vitro seedling explants of *E. flava* with 2-3 shoot buds differentiated from rhizome buds (**Figure 3.2**) were cultured in different culture systems containing MS medium supplemented with 150 ml/L CW, 50 g/L PE and 20 g/L sucrose. SSS system comprised of 20 ml medium in 4 oz. solidified with 7.5 g/L agar and 2 g/L activated charcoal whereas 125 ml conical flask containing 20 ml liquid medium were shaken on 110 rpm rotary shaker. SSS and CIS systems involved three replicates of 20 explants. Besides, TIS system modified from M Escalona et al. (1999) were set up and compared. Twenty explants were cultured in TIS vessel containing 400 ml liquid medium. One bottle of TIS set represented one replicate and three replicates were set up for this system. The liquid medium in TIS was feed for 5 minutes for every 4 hours. All culture systems were maintained under 12/12 hours light/dark photoperiod 40 µmol/m^2/s intensity of light

using warm-white LED lamp for 4 weeks. Survival rate, plant quality and quantity were observed and evaluated the propagation efficiency of different culture systems.

Figure 3.2 In vitro seedling of *Epipactis flava* was used as the initial explant in the experiment.

Greenhouse acclimatization and ex vitro cultivation

Healthy and vigorous plantlets of *E. flava* received from different culture systems were used for acclimatization and ex vitro cultivation. Thirty plantlets of *E. flava* were rinsed with running tap water to eliminate culture medium. Subsequently, they were transplanted into plastic pots containing mixed potting medium; Hydroton: Pumice (1: 1) (one plantlet per pot) and then acclimatized in a plastic box for 9 weeks. Plantlets were watered once a week and sprayed with liquid N-P-K fertilizer (20-20-20) every two weeks. After 9 weeks of acclimatization, plantlets were transferred to cultivate in artificial stream for further 8 weeks under greenhouse environment.

Experimental design and data analysis

All the experiments were conducted with complete randomize design (CRD). The difference of seed germination, seedling development, propagation efficiency in different culture systems and also transplantation were performed. Mean values of their observing parameters were statistically compared by one-way analysis of variance (ANOVA) followed by Duncan's new Multiple Range Test (DMRT).

Results

Effect of culture media on asymbiotic germination of *Epipactis flava*

The 6 WAP seeds from cross-pollination were cultured on different types of media in order to test germination efficiency. Different germination and protocorm development on each media were observed (**Figure 3.4**, **Table 3.1**). The quickest germination was found in BM and MM media. At 2 weeks after culture, the germination percentage on semi-solid BM and MM media were 20% and 12.4%, respectively, which were significantly higher than the other media (**Figure 3.4**, **left panel**). The germination percentages of these media were sharply increased (40-60%) up until the 4th week of culture and then slowly increased thereafter. Interestingly, although less than 3% germination percentage at 2 weeks after culture was noticed in VW media, the germination percentages were rapidly enhanced to 20-40% in the 4th week and over 60% in the 6th week of culture. This scenario was also observed in MS media but with lower enhancement rate (**Figure 3.4**, **left panel**). When considered the protocorms in advanced stages of development, protocorm in stage 4 or 5 were not observed in any media at 4 weeks after culture (**Figure 3.4**, **right panel**). Then, at 6 weeks after culture, they were greatly increased to approximately 40% in BM and liquid VW media, which were significantly greater than the other media. Surprisingly, at 8 weeks after culture, the percentage of advanced-stage protocorms was slightly increased in liquid BM medium, whereas they were considerably increased in semi-solid MM and VW. Consequently, the percentage of advanced protocorms in semi-solid MM, semi-solid BM semi-solid VW and liquid VW media were not significantly different at 8 weeks after culture (**Figure 3.4**, **right panel**). When compared between the semi-solid and liquid media system, the percentage of germination and advanced-stage protocorms were no significant different in all tested medium formulas except advanced protocorms of MM medium (**Figure 3.4**, **Table 3.1**). At the final observation, 2 months after culture, KC medium showed the germination percentage lower than 10% (**Table 3.1**). In addition, there were no stage 5 protocorms found in MS, KC and liquid MM media. Nonetheless, the highest percentage of germination (69.2%) and stage 5 protocorms (54.0%) were obtained from semi-solid BM medium. Therefore, the most appropriate medium for asymbiotic germination of *E. flava* was semi-solid BM medium.

Effect of culture media on in vitro culture of *Epipactis flava* seedlings

Although BM and MM media presented the good germination and protocorm development, the subsequent seedlings failed to grow in these media. Thus, in vitro seedling cultures were performed using VW and MS basal media which were widely used for orchid propagation. After 4 weeks of culture, the seedlings can be survived 100% and more than 96% of them can produce new shoots in all tested media (**Table 3.2**). The seedlings cultured on semi-solid media showed significantly lower growth performance than liquid media. The best overall growth performance was gained from liquid MS medium. Especially, shoot height (4.3 cm/shoot) and fresh weight (734.6 mg/plant) of seedlings cultured in this medium were significantly greater than the other media (**Figure 3.5**, **Table 3.2**).

Comparative study on efficient micropropagation system in *Epipactis flava*

The hundred percentage of survival rate of *E. flava* plantlets cultured in different systems; SSS, CIS and TIS could grow and proliferate new shoots and shoot buds and also indicated no hyperhydric symptoms after 4 weeks of culture (**Figure 3.6**). Regeneration and proliferation efficiency of explants cultured in 3 different culture systems showed obviously different and quality of regenerated plantlets could be classified into 3 different levels as illustrated in **Figure 3.7a**. The results revealed that the highest percentage of healthy and good growing plantlets was found in TIS (53.3%) followed by SSS (20.0%) and CIS (5.5%), respectively (**Figure 3.7b**). However, more than 50% of poor quality of plantlets were observed both in SSS and CIS. TIS had influenced on growth and development of *E. flava* higher than SSS and CIS systems. (**Table 3.3**). The highest percentage of new shoot formation (96.7%) and shoot bud formation (91.7%) could observe in TIS followed by SSS (46.7%) and CIS (40.0%), respectively (**Table 3.3**). In addition, TIS system was not only promoted the number of new shoots (1.5 shoot/explant), shoot buds (8.1 shoot buds/explant) and roots (4.4 roots/explant) but also stimulated shoot height (29.4 mm/shoot) and numbers of leaf (4.4 leaves/shoot) higher than SSS and CIS systems (**Table 3.3**). A comparison of the total number of new shoots and shoot buds obtained from one container or culture unit reveals that the use of the TIS system resulted in significantly

higher amounts of both new shoots (28.7) and shoot buds (161.0 shoot buds) than SSS and CIS systems (**Table 3.3**).

Acclimatization and ex vitro cultivation

Survival and growth of *E. flava* plantlets obtained from different culture systems were investigated under acclimatization condition. Complete hardening was established for 9 weeks. Different plant quality of *E. flava* were evaluated according to the following criteria; plant dead, plant survival with poor growth; plant with no new shoot or shoot bud formation, and plant survival with good growth; plant with new shoot or shoots bud formation (**Figure 3.8a**). The highest percentage of survival rate was observed in TIS (76.7%) whereas less than 50% of *E. flava* plantlets cultured on SSS and CIS could survive after the end of acclimatization (**Figure 3.8a**). In addition, the highest of survival plantlet showing good growth (38.9%) was found in TIS followed by SSS (28.9%) and CIS (23.3%), respectively. TIS was somehow provided higher percentage of poor growth plantlets (46.7%) than SSS and CIS systems (**Figure 3.8a**). The last cultivation step of *E. flava* plantlets was performed after acclimatization by transferring to grow in artificial stream under greenhouse environment for 8 weeks (**Figure 3.8b**). The results revealed that *E. flava* plantlets obtained from TIS had higher survival rate (60%) during cultivated into artificial stream than SSS (33.3%) and CIS (26.7%) systems, respectively (**Figure 3.8b**).

Discussions

Effect of culture media on asymbiotic germination of *Epipactis flava*

Asymbiotic germination and protocorm development of orchids were significantly affected by culture medium compositions (Dutra et al., 2009; Nanekar, Shriram, Kumar, & Kishor, 2014). Although orchid specific media like KC (Knudson, 1946), VW (Vacin & Went, 1949) and BM (Van Waes & Debergh, 1986) have been especially formulated, some orchid species did not germinate well in these media. The germination and protocorm development in each medium varies greatly form one orchid species to another. Thus, many germination media have been tested and modified in order to find the suitable media for seed germination of particular orchid species (Dutra et al., 2009; Johnson, Stewart, Dutra, Kane, & Richardson, 2007;

Pakum et al., 2016). From the results, at 4 weeks after culture, BM and MM media exhibited higher germination rates than the other tested media (**Figure 3.4**). The fast germination in BM and MM might be because both media contain casein hydrolysate, which has previously been reported that it can accelerate the germination of orchid seeds (Kumar, Nandi, Bag, & Palni, 2002; Mead & Bulard, 1975; Nanekar et al., 2014). Malmgren (1996) had discussed that young protocorms possibly metabolized organic nitrogen easier than inorganic nitrogen since amino acid in organic additive may detour some steps in the nitrogen assimilation pathways. Besides, BM contains high amount of manganese sulphate and it was previously reported that the germination of *Dactylorhiza* spp. seeds is depended on manganese sulphate (Vaasa & Rosenberg, 2004), so manganese sulphate may be one of the elements inducing the germination in *E. flava*. However, after 4 weeks of culture, the germination rate in BM and MM media was gradually raised whereas VW medium was sharply increased and equally effective to BM at 8 weeks after culture (**Figure 3.4**). It might be due to VW contains higher phosphate and ammonia contents compared to the other medium. Previously, it has been reported that orchid can uptake ammonium better than nitrate during germination (Dutra et al., 2008; Stewart & Kane, 2006) due to low activity of nitrate reductase in early protocorm development (Dutra et al., 2008; Van Waes & Debergh, 1986). In addition, Dutra et al. (2008) indicate that the higher phosphate concentration in VW medium promotes better seedling development in *Bletia purpurea.* In some plant species, phosphate in the culture medium is necessary for in vitro culture, because plant growth is inhibited when phosphate is depleted from the medium (George, Hall, & De Klerk, 2008). Addition of CW and PE in VW medium in this study may be one of the factors that enhanced protocorm development due to it contains various beneficial substances for seed germination, such as cytokinin, vitamins, amino acids and sugar (see review in Yong, Ge, Ng, and Tan (2009)).

Although liquid culture system provided the rapid growth and scaling up capability, it has occasionally been applied in orchid seed cultures because the complication of culture operation and high contamination rate. In addition, some orchid species failed to germinate under liquid culture system (Harvais, 1982). Nevertheless, *E. flava* is rheophytic orchid, so liquid medium is expected to be more efficient for seed germination than semi-solid medium. However, at the end of

observation, seed germination and protocorm development between liquid and semi-solid cultures in all tested basal media formulas were no significant differences except MM medium that the semi-solid cultures presented higher advanced-stage protocorms (stage 4 and 5) production compared to the liquid cultures (**Figure 3.4**). The same result was also observed in asymbiotic germination of *Doritaenopsis*, hybrids of *Phalaenopsis* and *Doritis* (Tsai & Chu, 2008). On the other hand, the germination rate of *Disa* spp. (Thompson, Edwards, & van Staden, 2006) and *Cypripedium debile* (Hsu & Lee, 2012) were higher in the liquid cultures than those found in the semi-solid cultures.

Effect of culture media on in vitro culture of *Epipactis flava* seedlings

Orchids require nutrients differently for each developmental stage (P. J. Kauth, Vendrame, & Kane, 2006). From the results, germinated seedlings of *E. flava* failed to grow further in BM and MM media although it showed good germination efficiency. Accordingly, BM and MM media were appropriate for seed germination rather than seedlings cultivation. Consequently, VW and MS basal media that widely used for orchid propagation (J. T. Silva, 2013) were employed for *E. flava* seedlings cultivation. Seedling of *E. flava* that cultured in liquid MS medium showed the best growth and multiplication (**Figure 3.5** and **Table 3.2**). This might be because MS medium is rich in both macro- and micro-nutrients required for plant growth and development (Stewart & Kane, 2006). Particularly, MS medium contains high contents of inorganic nitrogen in both nitrate and ammonium forms, which is necessary for growth and differentiation of plant cell (Hajong, Kumaria, & Tandon, 2010). Besides, liquid MS medium promoted better growth of *E. flava* seedling than semi-solid medium. It was may be due to plant can uptake nutrient faster and easier (M. Malik, Warchoł, Kwaśniewska, & Pawłowska, 2017). Furthermore, *E. flava* is rheophytic orchid so it may prefer more water content in the medium.

Comparative study on efficient micropropagation systems of *Epipactis flava*

The results indicated that TIS improved better growth and proliferation for mass propagation of *E. flava* than SSS and CIS. From the result, CIS was not recommended for large scale mass production of *E. flava* although this plant is

generally displaying a rheophytic habit. However, several former reports have revealed that different culture systems resulted in diverse growth and morphogenesis (Moreira et al., 2013; Vives et al., 2017; Yan, Liang, & Li, 2010). Some results showed that the highest ratio of normal plantlets was obtained in TIS treatment, whereas SSS and CIS treatment exhibited higher plant-abnormality rate (An et al., 2016; Etienne & Berthouly, 2002). This might be assumed that the SSS medium provided less nutrient absorption than TIS and CIS (M Escalona et al., 1999), while CIS frequently induce abnormality and necrosis symptoms due to the permanent immersion (R. V. Sreedhar et al., 2009).

Furthermore, TIS also showed better shoot and shoot bud proliferation as well as root and leaf induction number of *E. flava* than SSS and CIS. Relative results were also found in *Vanilla planifolia* (Ramos-Castellá, Iglesias-Andreu, Bello-Bello, & Lee-Espinosa, 2014) and *Rubus* spp. (Arencibia, Vergara, Quiroz, Carrasco, & García-Gonzales, 2013). An advantage of TIS over SSS and CIS are an alteration between aeration and periodically immersion of explants in liquid medium to improve gaseous exchange, increase an oxygen supply to explants in order to avoid the hyperhydration problem (C. Aragón et al., 2014; Berthouly & Etienne, 2005; Zhao et al., 2012). Furthermore, TIS could also eliminate some toxic gases i.e. ethylene during air feeding for immersion cycles (Georgiev et al., 2014), whereas mostly air ventilation are nearly not observed in close system like SSS and CIS. An enhancing of photomixotrophic culture could be observed during renewal atmosphere in TIS and also stimulate better growth and development of *E. flava* than SSS and CIS.

Acclimatization and ex vitro culture

Acclimatization is an important procedures to support the successful of plantlet transplantation from in vitro to ex vitro environment (Dohling, Kumaria, & Tandon, 2012; Hazarika, 2003). This process allows in vitro plantlets survived and adapted to natural environment of which normally has higher light intensity and lower humidity compared to in vitro environment (Hazarika, 2003). During acclimatization process, plantlets of *E. flava* obtained from TIS higher survival rate and successfully grew and adapted under environmental change. In contrast, morphological disorders of plantlet were mostly appeared in plantlets derived from SSS and CIS. Many

previous reports indicated that plantlets from TIS have greater growth and rapid adaptation after removed to ex vitro condition than SSS and CIS derived plant (An et al., 2016; S.-H. Yang & Yeh, 2008). This might be due to the fact that TIS could enhance stomatal functioning or improved photosynthesis and transpiration (Carlos Eduardo Aragón et al., 2010; Hazarika, 2003). However, plantlets of *E. flava* were visibly wilted during transferred to ex vitro acclimatization. Related reports found that wilting symptom caused by temporary dormancy during transplantation could be occurred in some species i.e *Eulophia cullenii* (Decruse, Reny, Shylajakumari, & Krishnan, 2013) and *Calopogon tuberosus* (P. Kauth, 2005). Survival plantlets of *E. flava* received from all culture systems at acclimatization step were transferred to cultivate into artificial stream for further 8 weeks. The results revealed that TIS derived plantlets gave the higher percentage of survival than that obtained from SSS and CIS, respectively.

Conclusions

Successful protocol for in vitro seed germination, essential factors affecting growth and development of *E. flava* seedlings as well as ex vitro transplantation process were studied and reported. Seeds obtain from 6 weeks after cross-pollination is a potential explant for asymbiotic germination. Semi-solid BM medium is the most suitable culture medium for seed germination and protocorm development, whereas liquid MS medium shows the greatest culture medium for stimulating growth and development of *E. flava* seedling. TIS is the most efficient and suitable method for mass propagation of *E. flava*. Moreover, plantlets obtained from TIS were successfully adapted and survived during acclimatization. Therefore, this practice is significantly improved and benefit to apply for ex vitro conservation of *E. flava*.

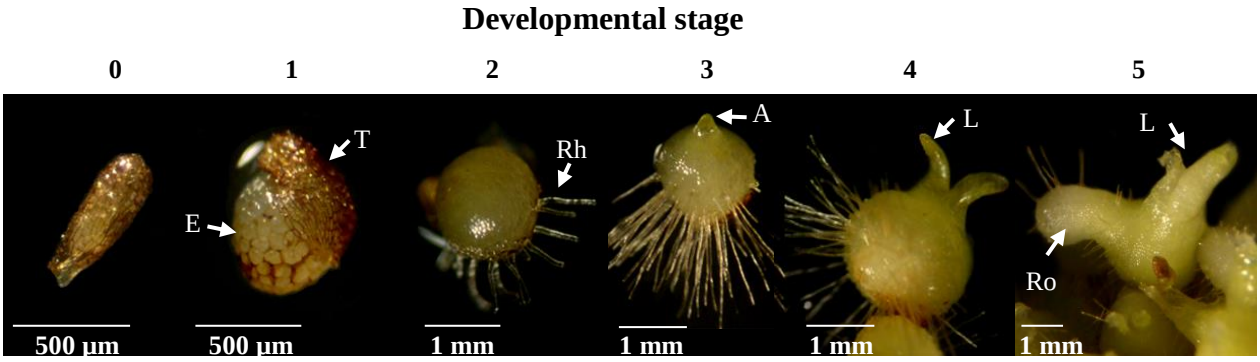

Figure 3.3 Developmental stages of asymbiotic germination of *Epipactis flava* seed. Stage 0, no development of seed. Stage 1, embryo (E) enlargement and rupture from the testa (T); Stage 2, appearance of rhizoids (Rh) at the basal of protocorm (germination); Stage 3, apical meristem (A) production; Stage 4, emergence of the first leaf (L); Stage 5, elongation of apical meristem and emergence of root (Ro).

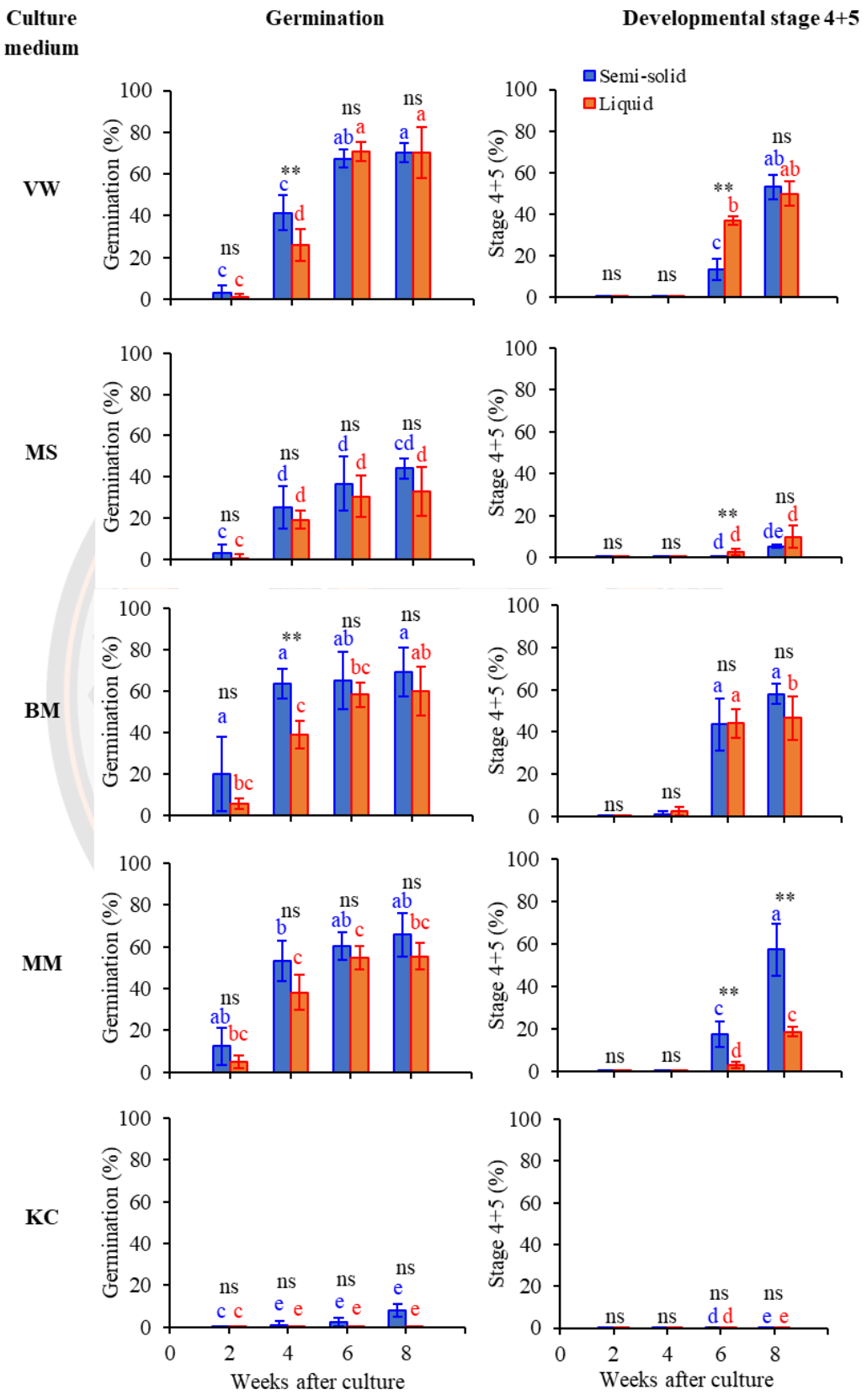
Culture medium
Germination
Developmental stage 4+5
Semi-solid
Liquid
VW
MS
BM
MM
KC
Germination (%)
Stage 4+5 (%)
Weeks after culture
0
2
4
6
8
0
20
40
60
80
100

Figure 3.4 Time course of percentage of germination (left) and protocorm developmental stage 4 plus 5 (right) of *Epipactis flava* asymbiotic seed germination on different culture media and state of medium. The same letter within the same weeks after culture of each observation parameter is not significantly different at $p \leq 0.05$ according to DMRT. The blue and red letters represent the DMRT analysis of semi-solid (blue bar) and liquid (red bar) culture system, respectively. The differences between semi-solid and liquid culture systems of each media at particular week according to independent t-test are represented by ns, non-significant difference; *, significant difference at $p \leq 0.05$ and **, highly significant difference at $p \leq 0.01$.

Table 3.1 Effect of culture media and state of culture on asymbiotic germination of *Epipactis flava* seeds (6 WAP) after 8 weeks of culture.

Media[1]	State of culture	Developmental stage (%)						Germination (%)[2]
		Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	
VW	Solid	27.4 ± 5.1 de	2.4 ± 1.7 b	2.0 ± 0.7 de	15.2 ± 8.1 bc	24.0 ± 5.2 ab	29.0 ± 4.2 c	70.2 ± 4.7 a
	Liquid	22.0 ± 8.5 e	7.6 ± 4.6 b	11.4 ± 6.8 b	9.2 ± 4.5 cd	11.4 ± 4.2 cde	38.4 ± 4.2 b	70.4 ± 12.2 a
MS	Solid	34.4 ± 5.0 cd	21.4 ± 4.3 a	19.8 ± 3.6 a	19.2 ± 3.3 b	5.2 ± 0.8 ef	0.0 ± 0.0 d	44.2 ± 5.0 cd
	Liquid	40.2 ± 8.4 c	26.6 ± 8.5 a	10.0 ± 3.7 bc	13.4 ± 10.6 bcd	9.8 ± 5.4 de	0.0 ± 0.0 d	33.2 ± 11.8 d
BM	Solid	24.8 ± 9.9 de	6.0 ± 3.7 b	4.4 ± 5.9 cde	6.8 ± 4.6 de	4.0 ± 5.5 ef	54.0 ± 7.4 a	69.2 ± 11.6 a
	Liquid	34.2 ± 12.3 cd	6.0 ± 2.5 b	4.6 ± 5.2 cde	8.6 ± 0.9 cd	13.2 ± 11.1 cd	33.4 ± 11.5 bc	59.8 ± 11.8 ab
MM	Solid	32.4 ± 10.0 cde	1.8 ± 1.3 b	1.2 ± 1.8 e	7.2 ± 3.3 de	30.2 ± 9.0 a	27.2 ± 12.8 c	65.8 ± 10.4 ab
	Liquid	41.0 ± 5.1 c	3.6 ± 2.9 b	10.2 ± 5.6 bc	26.6 ± 5.4 a	18.6 ± 2.3 bc	0.0 ± 0.0 d	55.4 ± 6.3 bc
KC	Solid	68.8 ± 4.8 b	23.0 ± 4.8 a	7.6 ± 2.7 bcd	0.4 ± 0.9 e	0.2 ± 0.4 f	0.0 ± 0.0 d	8.2 ± 3.1 e
	Liquid	86.0 ± 5.5 a	14.0 ± 5.5 b	0.0 ± 0.0 e	0.0 ± 0.0 e	0.0 ± 0.0 f	0.0 ± 0.0 d	0.0 ± 0.0 e

Values are mean ± SD of 5 replicates (100 seeds per replicate). The same letters within a column are not significantly different at $p \leq 0.05$ according to DMRT.

[1] VW, modified Vacin and Went medium; MS, Murashige and Skoog medium; BM, BM terrestrial orchid medium; MM, Malmgren modified terrestrial orchid medium; KC, Knudson's C medium.

[2] Germination percentage of *E. flava* was calculated from developmental stage 2 to 5.

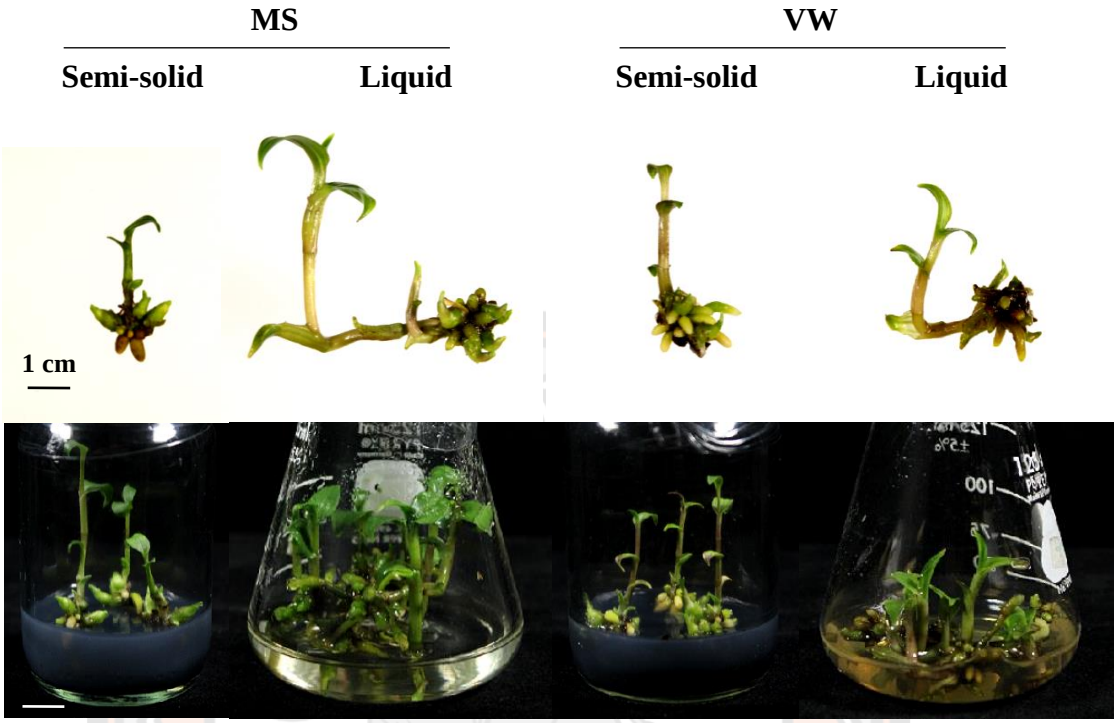

Figure 3.5 Growth and development of *Epipactis flava* seedling after culture on semi-solid or liquid states of MS or VW medium for 8 weeks.

Table 3.2 Effect of culture media and state of culture on growth and development of *Epipactis flava* seedlings after 8 weeks of culture.

Media	State of culture	Survival (%)	Shoot				Root		Fresh weight[3] (mg)
			NSF[1] (%)	Number	Height (cm)[2]	Width (mm)[2]	Number of laves[2]	Number	Length (cm)	
MS	Semi-solid	100	100.0	3.7 ± 0.6 b	2.2 ± 1.1 c	2.0 ± 0.2 b	1.3 ± 0.7 b	3.4 ± 1.2 b	1.0 ± 0.3 ns	379.5 ± 82.8 c
	Liquid	100	96.7	4.5 ± 1.3 a	4.3 ± 1.0 a	2.1 ± 0.3 ab	1.9 ± 0.7 a	5.1 ± 3.3 a	0.9 ± 0.4	734.6 ± 52.3 a
VW	Semi-solid	100	100.0	4.1 ± 0.7 ab	2.2 ± 0.5 c	2.0 ± 0.5 b	1.6 ± 0.6 ab	6.2 ± 3.1 a	0.9 ± 0.6	428.3 ± 49.1 c
	Liquid	100	96.7	4.0 ± 1.1 ab	3.0 ± 0.6 b	2.2 ± 0.4 a	2.0 ± 0.7 a	5.3 ± 2.1 a	1.0 ± 0.4	618.4 ± 87.1 b

Values are mean ± SD of 15 plants. The same letters within the column are not significantly different at $p \leq 0.05$ according to DMRT.

[1] NSF; new shoot formation.

[2] Data were derived from the highest shoot of each plant clumps.

[3] Fresh weight per clump

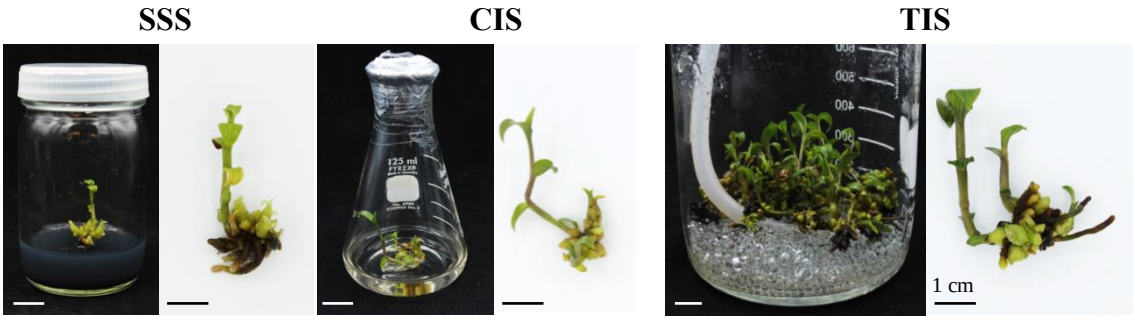

Figure 3.6 Growth and development of *Epipactis flava* plantlets at 4 weeks after cultured in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) culture systems.

Table 3.3 Effect of culture systems on growth and development of *Epipactis flava* plantlets at 4 weeks after culture in MS medium.

Parameters	Culture systems [1]		
	SSS	CIS	TIS
Survival rate (%)	100.0 ± 0.0 ns	100.0 ± 0.0	100.0 ± 0.0
New shoot formation (%)	86.7 ± 0.6 b	70.0 ± 1.1 c	96.7 ± 1.3 a
Number of new shoots per explant	1.0 ± 0.0 b	0.8 ± 0.2 b	1.5 ± 0.1 a
Number of new shoot per container	1.0 ± 0.1 b	0.8 ± 0.1 b	28.7 ± 9.5 a
Shoot bud formation (%)	46.7 ± 1.3 b	40.0 ± 1.1 b	91.7 ± 1.7 a
Number of shoot buds per explant	3.9 ± 0.1 b	5.5 ± 0.2 b	8.1 ± 0.4 a
Number of shoot bud per container	3.9 ± 0.3 b	5.5 ± 0.7 b	161.0 ± 15.0 a
Number of roots per explant	3.8 ± 0.0 ab	3.6 ± 0.0 b	4.4 ± 0.1 a
Number of leaf per shoot [2]	2.8 ± 0.1 b	2.7 ± 0.5 b	4.4 ± 0.1 a
Shoot height (mm) [2]	14.0 ± 0.4 b	14.3 ± 3.4 b	29.4 ± 0.8 a

Values are mean ± SE of 3 replicates (20 explants per replicate) except number of new shoot and shoot bud per container are mean ± SD of 3 containers. The same letter within a row is not significantly different at $p \leq 0.05$ according to DMRT.

[1] SSS, Semi-solid system; CIS, Continuous immersion system and TIS, Temporary immersion system.

[2] Leaf number and shoot height were recorded from the longest shoot length of each explant.

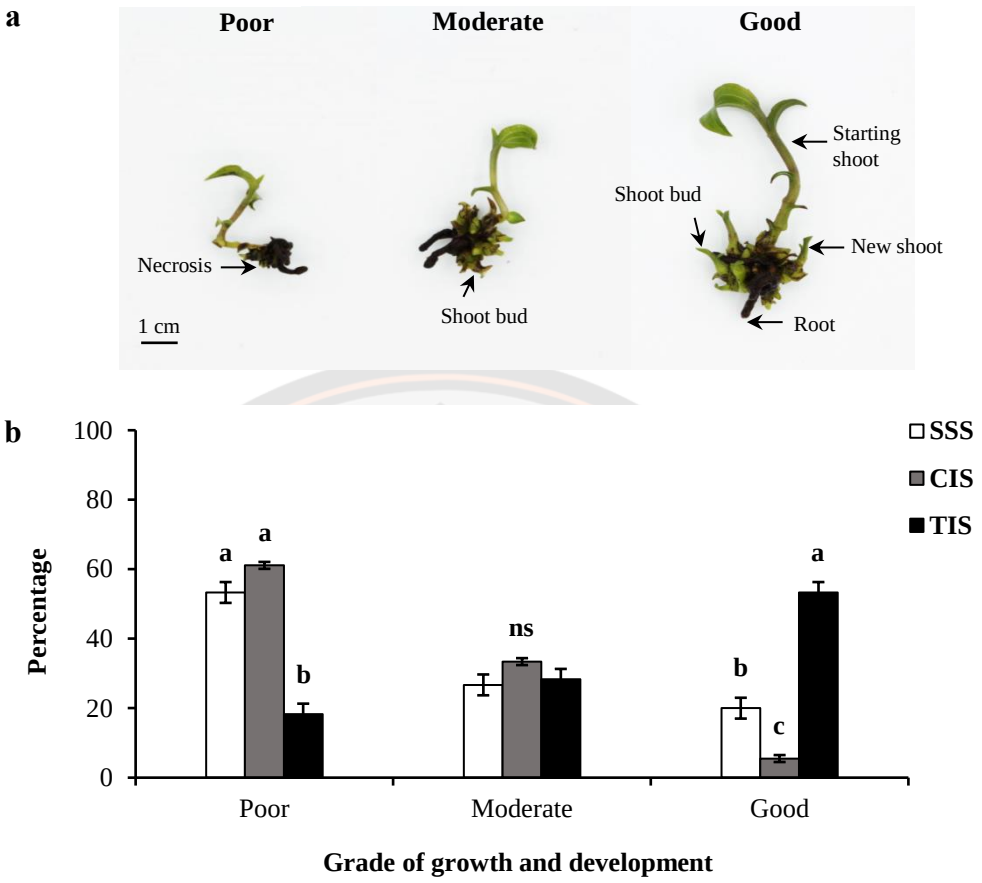

Figure 3.7 Classifying system for measuring growth and development of *Epipactis flava* plantlets (a) and comparative effects of culture systems on growth and development grade of *E. flava* plantlets after 4 weeks of culture (b). The results represent mean of 3 replicates each 20 explants. The error bars indicate standard error (SE). The different letter within the same grade of growth and development is significantly different at $p \leq 0.05$ according to DMRT. Grading criteria: Good; new shoots and shoot buds formation, Moderate; shoot bud formation, Poor; tissue necrosis or no shoot bud formation.

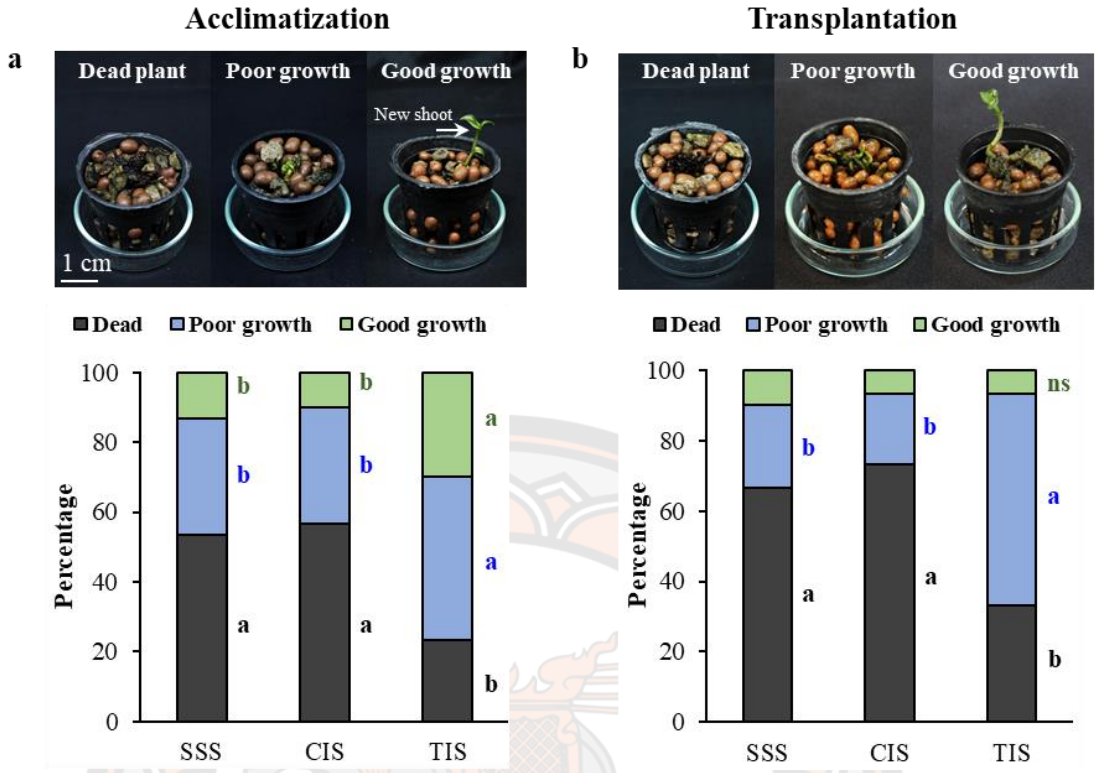

Figure 3.8 Plant quality and growth of *Epipactis flava* after acclimatization for 9 weeks (a) and after transplantation within artificial stream for 8 weeks (b). Different criteria of quality were assessed dividing to dead plant, plant survival with poor growth; plant with no new shoot or shoot bud formation, and plant survival with good growth; plant with new shoot or shoots bud formation. The results represent mean of 3 replicates (10 plants per replicates). The same letter of each observation parameter is not significantly different at $p \leq 0.05$ according to DMRT.

CHAPTER IV

Mass Propagation of *Calanthe rubens* Ridl., an Ornamental Orchid: A Comparison Between Semi-solid, Continuous Immersion and Temporary Immersion Systems

Summary

Calanthe rubens is a native terrestrial orchid distributed in Southeast Asia. Among the current situation, many orchid plants including *Calanthe* spp. have been affected from environmental change. In order to establish the effective protocols for plant propagation, the in vitro cultivation system of *C. rubens* was used to compare potentiality. In vitro pseudo-bulblets of *C. rubens* were grown in three culture systems; semi-solid system, continuous immersion and temporary immersion system. After 8 weeks of cultivation, The results found that the highest plant survival rate could obtain from SSS (97.2%) followed by TIS (95%) and CIS (79.2 %), respectively. However, 100% of new shoot formation was observed only in CIS and new regenerated shoots derived from explants cultured in all systems showed no hyperhydricity. Moreover, the highest number of new shoots (2.3 shoots), roots (4.3 roots), leaf (2.8 leaves) and pseudo-bulblet diameter (0.43 cm) could obtain from CIS. In transplantation step, the highest percentage of survival was somehow found in plantlets derived from SSS.

Introduction

Orchidaceae, a largest family of flowering plants, has consisted of more than 20,000 species distributed around the world (Cribb et al., 2003). Interestingly, the orchids are scrutinized as one of the most fascinating groups of ornamental plants. Moreover, numerous species of orchid have been produced by hybridization with exotic and elegant flowers (Shin, Baque, Elghamedi, Lee, & Paek, 2011). The genus *Calanthe*, consists of approximately 200 species, is popular ornamental plants because of the wide range of variations in the attractive colors, pleasing fragrance and big flowers (Godo, Komori, Nakaoki, Yukawa, & Miyoshi, 2010). *Calanthe rubens* is

native orchid to Southeast Asia; Thailand, Philippines and Malaysia. This orchid has been found in several locations in southern and eastern regions of Thailand except the Northern regions. *C. rubens*, a terrestrial or lithophytic herbs, is habited on rocks in evergreen forest, often associated with limestone and sometimes on the edge of evergreen swamp forest from 800 meters above sea level. Flowering period of this plant begins from December to February (Kurzweil, 2010). However, generally plant in family of Orchidaceae have been susceptible to environmental disruption, succession of natural habitats and also overexploitation for horticultural processes. Regardless of this plants are high reproductive value but it is difficult to provide high quantity production in short period. In facts, the germination rate of *Calanthe* plants is very low (2%) in wildlife, which that leading to become quiescent and eventually disappear within 2-3 years (Shin et al., 2011). In order to overcome the limitations, it is necessary to establish the effective protocols for plant propagation with using the in vitro culture. Plant tissue culture technique has been frequently known as effective methods for propagation of economic plant (Esyanti, Adhitama, & Manurung, 2016; Ramírez-Mosqueda & Iglesias-Andreu, 2016), ornamental plant (Moreira et al., 2013; Shin et al., 2011), medicinal plant (Zhu et al., 2018) and also applied to plant conservation (Diengdoh et al., 2017). However, plants produced from this technological process using conventional culture systems are often hampered by a low number of propagation individuals per explant, high production cost and physiological disorder (K. Y. Paek et al., 2005). Consequently, one way to avoid the problems was modification to the liquid medium by using temporary immersion systems (TIS). TIS, involved flooding of plant tissue at regular time intervals, are developed to increase the multiplication rate of plant, reduce cost and labour requirements in commercial plant production (Etienne & Berthouly, 2002; Georgiev et al., 2014; Sharma et al., 2015). Thus, an improved protocol for *C. rubens* production using TIS may be an alternative methods for in vitro mass production. The aim of this research was to investigate the effect of culture systems on in vitro mass propagation efficiency of *Calanthe rubens*.

Materials and Methods

Preparation of plant materials

Calanthe rubens obtained from in vitro culture were used to investigate and compare propagation efficiency of different types of culture systems. About 1 cm of *C. rubens* pseudo-bulblets were used as starting explants (**Figure 4.1a**). Their leaves and roots of pseudo-bulblet were removed before cultured onto semi-solid Murashige and Skoog (MS) (1962) medium supplemented with 30 g/L of sucrose, 0.2 g/L myo-inositol, solidified with 2.0 g/L gelite and adjusted pH to 5.8 prior to autoclaving. The explants were maintained on this medium for week in order to observe and solve contamination problem (**Figure 4.1b**).

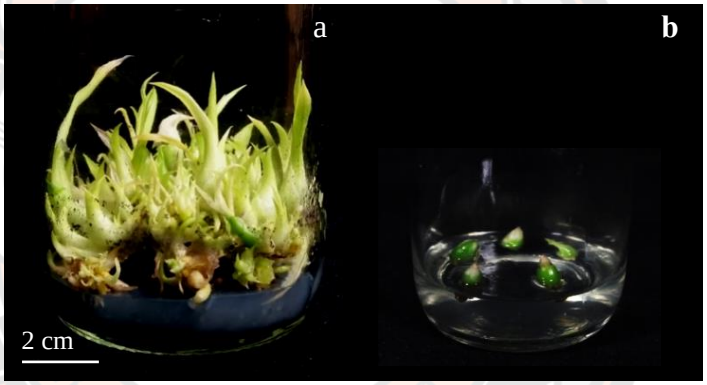

Figure 4.1 Pseudo-bulblets of *Calanthe rubens* were separated from in vitro stock plantlets (a). Leaves and roots of pseudo-bulblets were removed before cultured on MS semi-solid medium for 1 week. These explants were then used as starting materials for the experiment (b).

Mass propagation of *Calanthe rubens* in semi-solid, continuous immersion and temporary immersion systems

The influence of the culture systems on propagation efficiency of *Calanthe rubens* cultivation was observed and compared in semi-solid system (SSS), continuous immersion (CIS) and temporary immersion system (TIS). In this experiment, MS basal medium was used as propagating medium, supplemented with 30 g/L sucrose and 0.2 g/L myo-inositol. The pH of medium was adjusted to 5.8 using 1 N NaOH or HCl and autoclaved at 121 ^{o}C for 15 min before use. The pseudo-

bulblets of *C. rubens* obtained from previous procedure were cultured on semi-solid or continuous immersion and temporary immersion system. Each culture method was performed with three replicates. For SSS, 20 ml of medium solidified with 2.0 g/L gelrite were filled in 4 oz glass bottle whereas 20 ml of liquid medium filled in 125 mL conical flask were set for CIS and culture on 110 rpm rotary shaker. Four explants per culture vessel of SSS and CIS were employed. For TIS treatment, 1 L twin-bottles supported by small glass beads was used. Twenty of *C. rubens* pseudo-bulblets were cultured in each TIS vessel containing with 400 mL liquid medium of the same composition. The cultures were immersed into liquid medium for 5 min in every 4 hours. This culture system was designated by one vessel per replicate. All 3 culture systems were maintained in growth room at of 25±2 ºC under warm-white LED (40 µmol/m^2/s of light intensity) for 12 hours of photoperiods. After 8 weeks of cultivation, growth parameters such as survival rate, new shoot formation, number of shoots, leaf length and diameter size of new pseudo-bulblets measured by vernier caliper, were observed, calculated and compared.

Transplantation of *Calanthe rubens* in greenhouse condition

Individual plantlet obtained from SSS, CIS and TIS were transplanted into pot (2x2 inch) containing potting medium mixture of hydroton and pumice (1: 1) and then kept in greenhouse conditions. Plantlets were watered by spraying every 2 weeks. Transplantation of *C. rubens* were performed with 3 replicates and each replicate contains 10 plantlets. The data Growing data was observed and recorded and survival rate of plantlet derived from each culture system was calculated and compared after 8 weeks of cultivation.

Experimental design and data analysis

Complete randomized design was used in all experiments. Different mean values of observing parameters were statistically compared by one-way analysis of variance (ANOVA) followed by Duncan's new Multiple Range Test (DMRT) at $p \leq 0.05$.

Results

Mass propagation of *Calanthe rubens* through semi-solid, continuous immersion and temporary immersion systems

In vitro pseudo-bulblets of *Calanthe rubens* cultured different systems in displayed diverse features. However, plantlets regenerated in all culture systems showed no sign of hyperhydricity (**Figure 4.2**). The high survival rate of *C. rubens* pseudo-bulblets (> 75%) had been noticed in all culture systems. The highest survival percentage was somehow significantly found in SSS (97.2%) and TIS (95.0%) (**Figure 4.2**). The formation of new shoots from pseudo-bulblets could be divided into three types according to **Figure 4.3.** Three different of morphological responses: NSF (no new shoot formation), SF-I (new shoot regenerated from either basal or apical of pseudo-bulblets) and SF-II (new shoot regenerated from both basal and apical of pseudo-bulblets) were observed and different percentage of each shoot formation type was noticed. The result showed that 100% of new shoot formation from pseudo-bulblets of *C. rubens* were found in CIS. On the contrary, more than 20% of NSF was only found in both SSS and TIS. Furthermore, more than 50% of SF-I shoot formation type could be observed in all culture systems. However, SF-II shoot formation type in all culture systems was presented less than 30% and also showed no significant difference (**Figure 4.3**). Several statistically significant differences were observed in quantitative data (**Table 4.1**), a number of new shoots per explant (2.3 shoots) was 2 times higher in CIS than in SSS (1.1 shoots) and TIS (1.1 shoots) whereas the highest of total shoot number per container was found in TIS (21.3 shoots). In addition, CIS clearly increased a number of root and leaf per explant (4.3 roots and 2.8 leaves) higher than SSS and TIS. However, the highest mean leaf length regenerated from pseudo-bulblets derived shoots of *C. rubens* was nearly 2 times higher in CIS (5.5 cm) than in TIS (2.8 cm). Furthermore, the largest of pseudo-bulblet as indicated by diameter could be only observed when explants were cultured in liquid medium (CIS and TIS) (**Table 4.1**).

Ex vitro culture of *Calanthe rubens* in greenhouse condition

Plantlets of *C. rubens* obtained from SSS, CIS and TIS were transplanted and grew into potting medium under greenhouse condition for 8 weeks. The results

indicated that increasing growth and shoot formation of *C. rubens* plants were observed (**Figure 4.4** and **Table 4.2**). A hundred percent of survival rate were found in SF-I + SF-II plantlets from SSS while the lowest percentage of plantlet survival was detected in SF-I + SF-II from TIS (30%). However, the pseudo-bulblets with shoot transferred to ex vitro culture were showed less new shoot formation (**Table 4.2**). However, NSF of in vitro plantlets obtained from TIS did not propagate shoot formation (**Table 4.2**).

Discussions

The beneficial utilization of temporary immersion system for enhancing growth, improving plant quality and increasing multiplication rate has been frequently reported by many researchers (Mosqueda Frómeta et al., 2017; M. d. A. Silva, Medeiros, Lima, Willadino, & Camara, 2015; B. Zhang et al., 2018). From the investigation, new plantlets could not be produced from *C. rubens* pseudo-bulblets. In some reports, low multiplication rate and vigorous plantlet could be obtained from TIS may be due to some culturing explants or organs were sensitive to shear stress, that occurred from air bubble during immersion phase. The temporary flooding in TIS could create different forms of stress, such as temporary hypoxia, that may induce physiological disorders (Dewir, Chakrabarty, Ali, Hahn, & Paek, 2006; Fukao & Bailey-Serres, 2004). Mendonça et al. (2016) used different culture systems for in vitro production of *Eucalyptus camaldulensis* and found that the highest multiplication rate was presented in semi-solid medium and continuous immersion whereas TIS provided lower multiplication rate. Furthermore, many factors have been involved in production efficiency of TIS i.e. the immersion times, the duration and frequency are the most decisive parameter for a successful propagation (Georgiev, Ilieva, Bley, & Pavlov, 2008; Watt, 2012). Hence, these factors could be optimized and plant quantity and quality of *C. rubens* using TIS may then be improved. Contrary in this case that continuous immersion of *C. rubens* exhibited the most suitable culture system to improve shoot multiplication rate, which was probably related to extended immersion accelerated nutrient uptake of plantlets (Hahn & Paek, 2005). Previous reports revealed that liquid culture systems have significant effects on the multiplication rates and morphology of shoots, microtubers or pseudo-bulblets

produced in vitro (Preil, 2005). The diameter of pseudo-bulblet as storage organ of *C. rubens* was greatly enlarged in CIS and TIS, which probably due to continual of the explants with liquid medium, promoting water absorption in these systems. Similar results in biomass accumulation were observed in *Lessertia* plantlets (Shaik, Dewir, Singh, & Nicholas, 2010). However, temporary immersion system is still important for improving the multiplication rate and yield and reducing cost with using less usage space and saving energy when the commercial production need to be concerned.

In transplantation, high of survival rate was presented in plantlets obtained from SSS, whereas plantlets grown in liquid medium; CIS and TIS, showed poor survival and new shoot formation rates when transfer to natural environment. This probably due to the excessive water loss of plantlets during transplanting. Previous reports indicated that the plantlet of *Cattleya walkeriana* cultured by liquid medium showed the fastest loss of water content within few minutes and less than 10% of total water content was detected after transfer to ex vitro culture (Moreira et al., 2013).

Conclusions

The present study demonstrated the effect of culture systems on in vitro propagation of *Calanthe rubens* using pseudo-bulblet explants. The most effective culture system was CIS (continuous immersion system) for shoot multiplication per explant, leaf number and leaf length whereas TIS (temporary immersion system) with 5 min immersion times for once every 4 hours may be not suitable condition for growth and development of *C. rubens* using pseudo-bulblet explants. On the other hand, the highest of survival rate and new shoot formation of *C. rubens* plantlets could obtain from SSS (semi-solid system) after ex vitro transplantation.

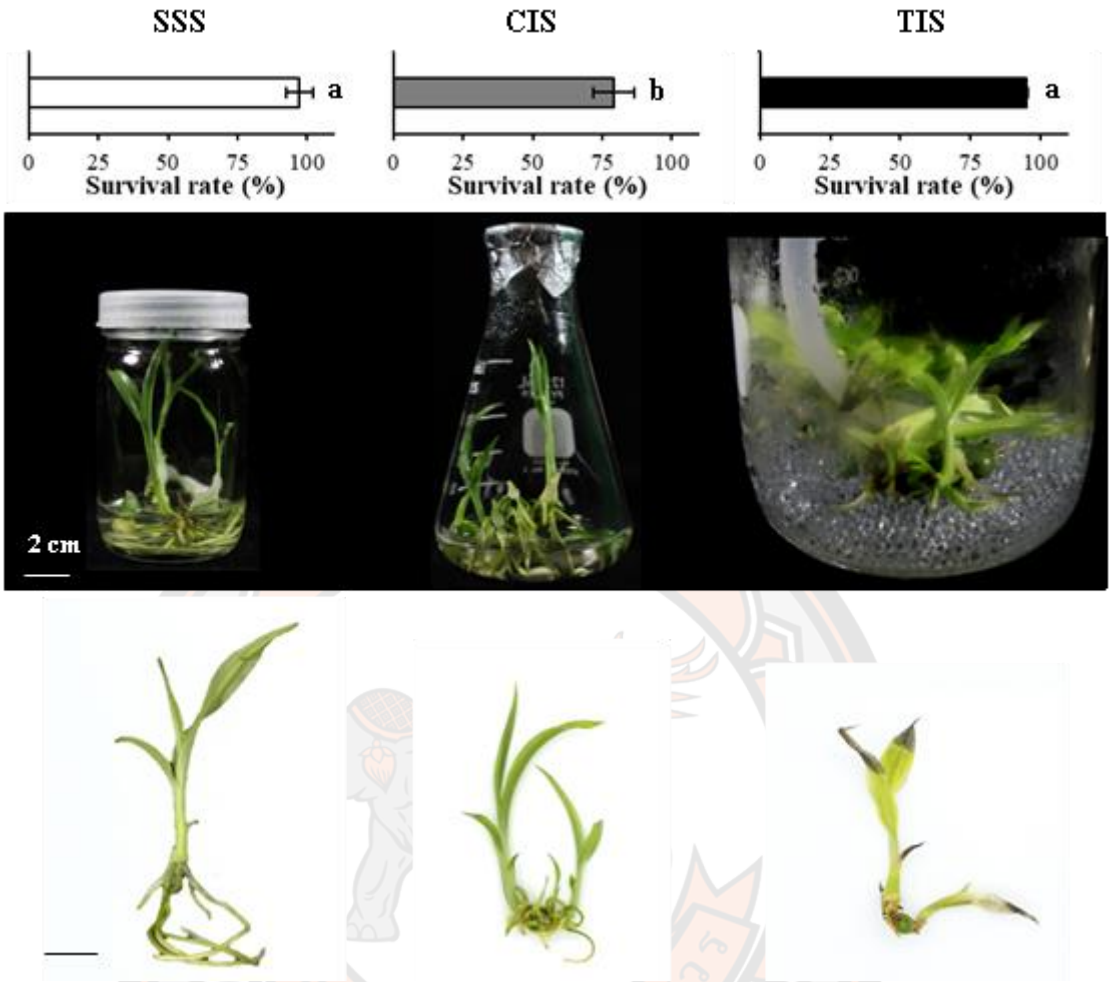

Figure 4.2 Comparative survival, growth and development of *Calanthe rubens* pseudo-bulblets after 8 weeks of cultivation in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) systems. The survival percentages are mean ± SD of 3 replicates. The same letters beside the bars indicate no significant difference of mean at $p \leq 0.05$ according to DMRT.

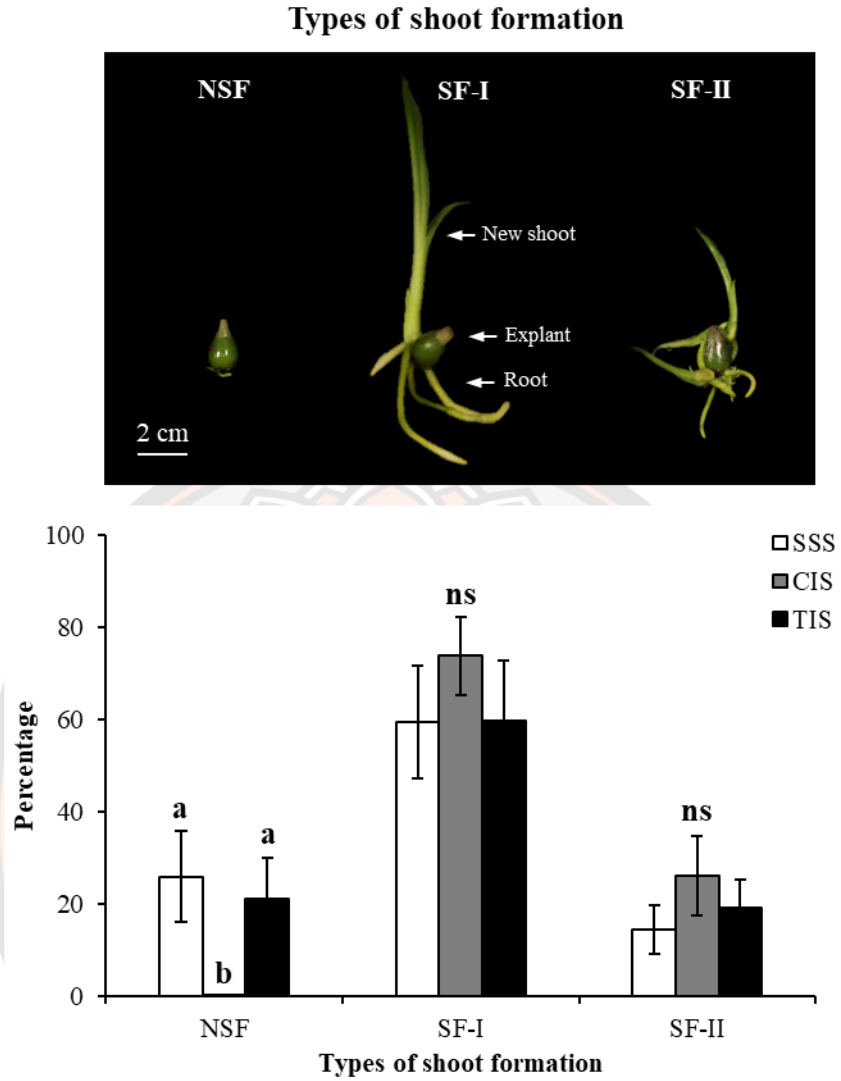

Figure 4.3 Types of shoot formation of *Calanthe rubens* pseudo-bulblets after 8 weeks of culture. The classification was sorted according to new shoot formation: NSF, no new shoot; SF-I, one new shoot and SF-II, two new shoots. Values are mean ± SD of 3 replicates. The same letters within the column are not significantly different at $p \leq 0.05$ according to DMRT.

Table 4.1 Growth and development of *Calanthe rubens* pseudo-bulblets after 8 weeks of culture in semi-solid system; (SSS), continuous immersion system; (CIS) and temporary immersion systems (TIS).

Growth parameters	Culture systems		
	SSS	CIS	TIS
Number of new shoot per explant	1.10 ± 0.10 b	2.30 ± 0.50 a	1.10 ± 0.00 b
Number of new shoot per container	4.40 ± 1.90 b	7.30 ± 2.30 b	21.30 ± 0.60 a
Number of root per explant	3.50 ± 0.60 a	4.30 ± 0.50 a	1.70 ± 1.00 b
Number of leaf per explant	2.00 ± 0.20 b	2.80 ± 0.10 a	1.80 ± 0.30 b
Leaf length (cm)[1]	4.21 ± 0.94 ab	5.50 ± 0.62 a	2.82 ± 1.08 b
New pseudo-bulblet diameter (cm)[2]	0.36 ± 0.02 b	0.43 ± 0.02 a	0.38 ± 0.04 a

Values are mean ± SE of 3 replicates, except number of shoot per container are mean ± SD of 3 containers. The same letters within a row are not significantly different at $p \leq 0.05$ according to DMRT.

[1] Leaf length were recorded from the longest leaf of pseudo-bulblet.

[2] Diameter of new pseudo-bulblet was measured from entire new pseudo-bulblets of each explant.

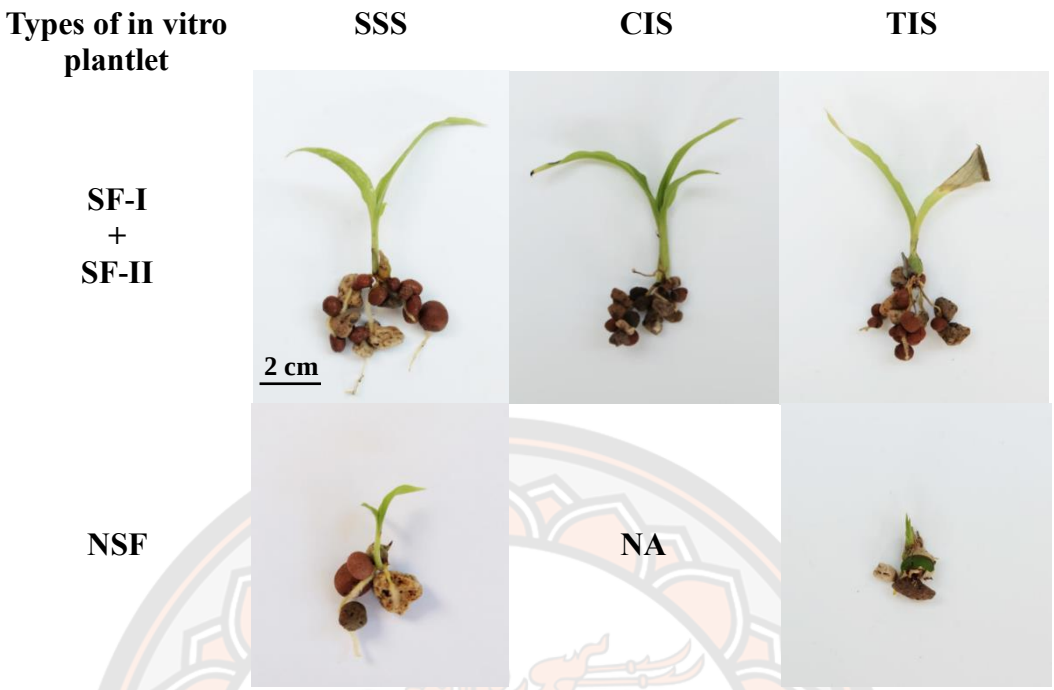

Figure 4.4 Growth of *Calanthe rubens* plant obtained from different in vitro culture systems after transplantation to greenhouse for 8 weeks. Two groups of in vitro plant were transplanted, pseudo-bulblets with shoots (SF-I and SF-II, **Figure 4.3**) and pseudo-bulblets without shoots (NFS, **Figure 4.3**), NA, not available

Table 4.2 Effect of culture systems on growth and survive of *Calanthe rubens* plantlets after 8 weeks of acclimatization under greenhouse condition.

Culture systems [1]	**Type of in vitro plantlet** [2]	**Survival (%)**	**New shoot formation (%)**
SSS	SF-I + SF-II	100.0 ± 0.0 a	10.3 ± 9.0 b
	NSF	81.1 ± 1.9 b	50.0 ± 10.0 a
CIS	SF-I + SF-II	42.1 ± 8.4 d	10.3 ± 9.0 b
	NSF	NA	NA
TIS	SF-I + SF-II	30.0 ± 8.7 d	0.0 ± 0.0 b
	NSF	58.3 ± 14.4 c	4.2 ± 3.2 b

NA, not available

[1] SSS, Semi-solid system; CIS, Continuous immersion system and TIS; Temporary immersion system.

[2] As described in **Figure 4.3**.

CHAPTER V

Comparison of Semi-solid, Continuous Immersion and Temporary Immersion Systems for Biomass and Plumbagin Production of *Drosera communis* A.St.-Hil., A Medicinal Plant

Summary

Drosera communis is a carnivorous plant with ornamental and medicinal values. The productions of biomass and plumbagin, a main bioactive compound in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) systems were performed and compared. After pre-induction leaves were cultured for 8 weeks, TIS obviously promoted better growth and development when compared to SSS and CIS. The highest biomass per clump (3.40 g FW and 0.36 g DW) was obtained from TIS, which was about 3- and 1.8-time higher than SSS and CIS, respectively. The highest shoot number per clump (30.4 shoots) was also derived from TIS which was around 2.6- and 1.6-fold over SSS and CIS, respectively. Moreover, the growth performances per culture container of TIS were enormously higher than SSS and CIS. Over 600 shoots were obtained from one TIS while only 23 and 38 shoots were obtained from SSS and CIS, respectively. Likewise, the maximal biomass per container (68.01 g FW and 7.09 g DW) was obtained from TIS which was greater than SSS and CIS up to 31 and 18 folds, respectively. For plumbagin production, the highest plumbagin content was gained from CIS (4.91 µg/g DW) followed by SSS (4.22 µg/g DW) and TIS (2.44 µg/g DW). Although TIS provides the lowest plumbagin content, the highest plumbagin yield (17.30 µg/container) that calculated per container was gained from TIS which was approximately up to 12.2 and 6.4 times higher than those of SSS and CIS, respectively. This study revealed that TIS is suitable for large-scale production of biomass and plumbagin of *D. communis*.

Introduction

The genus *Drosera* L. (Droseraceae) is the large group of carnivorous plants including more than 200 species distributed around the world except deserts and

Antarctic regions (Miclea & Zahan, 2017). Most of *Drosera* plants are found in open, wet, nutrient poor areas, especially low nitrogen and phosphorous soil (Rice, 2010). Therefore, the leaves of *Drosera* developed to become sticky traps for capture and digest insect prey to obtain the nutrients. *Drosera* plants have gained popularity as alternative ornamental plants (Barthlott & Ashdown, 2007) and folk herbal medicines to treat various illnesses, for instant, traumatic injury and detoxifying (Luo et al., 2018), corns (Prashanth Kumar & Shiddamallayya, 2016), bronchial asthma (Leporatti & Ivancheva, 2003; Mamedov & Craker, 2001) and dysentery (Vaidyanathan et al., 2013). The main secondary metabolite produced from these plants is a naphthoquinones, plumbagin. This bioactive compound exert a wide range of physiological and pharmacological activities, including anticancer (De et al., 2019; Yu et al., 2018), antimicrobial (Nair et al., 2016), antimalarial (Likhitwitayawuid, Kaewamatawong, Ruangrungsi, & Krungkrai, 1998) and antifungal activities (Tian et al., 2014)

In the past decades, the Droseraceae have become increasingly rare and therefore preserved in several counties (Stefano & Santos Silva, 2001; C. h. Wawrosch, Kongbangkerd, Kpf, & Kopp, 2005). *Drosera communis* is one of Droseraceae that was collected in large quantities for pharmaceutical purposes (Kongbangkerd, Homchan, & Promthep, 2011). In term of pharmaceutical property, some research indicated that *D. communis* had better efficiency of antimicrobial activity than other testing species (Ferreira et al., 2004). Moreover, some study revealed that in vitro shoot of *D. communis* provide higher the naphthoquinone content than greenhouse or outdoors growing shoot (C. h. Wawrosch et al., 2005). Therefore, in vitro shoot of *D. communis* may serve as alternative source of plumbagin production that can reduced over harvesting other Droseraceae from the natural populations.

Plant tissue culture technology has been used as alternative cultivation methods for mass propagation of various medicinal plants including *Drosera* (Jayaram & Prasad, 2007; Miclea & Zahan, 2017; Putalun et al., 2010). It has been disclosed that these technologies provide rapid clonal micropropagation and increase the yield of some secondary metabolites (Georgiev et al., 2008; Isah, 2015; Isah et al., 2017). However, different types of culture system have a different influence on the

propagation and bioactive compound production efficiencies (S. Malik, Hossein Mirjalili, Fett-Neto, Mazzafera, & Bonfill, 2013; Pérez-Alonso et al., 2009; Wilken et al., 2005). The conventional culture systems like semi-solid (SSS) and continuous immersion (CIS) systems are laborious with a number of handling steps. In addition, they have been often counterbalanced by some limitations such as slow growth and low multiplication rate in SSS, as well as hyperhydricity, abnormal morphology and asphyxia in CIS. (Etienne & Berthouly, 2002; Gao et al., 2015; Mallón, Covelo, & Vieitez, 2011). These problems increase the overall production cost especially in large-scale propagation. Alternatively, temporary immersion system (TIS) has been developed to retain good multiplication rate of CIS and high oxygen exchange of SSS. It is feasible to scale-up so appropriate for mass propagation (Pérez et al., 2013). A wide variety of medicinal plants such as *Habanero pepper* (Bello-Bello et al., 2010), *Leucojum aestivum* (Ivanov, Georgiev, Georgiev, Ilieva, & Pavlov, 2011) and *Stevia rebaudiana* (Vives et al., 2017) have been successfully propagated using TIS. However, in vitro culture using TIS of *D. communis* has not reported yet. Therefore, in vitro mass propagation of *D. communis* by TIS has been developed in this study. The efficiencies of TIS in the term of mass propagation, biomass and plumbagin productions were compared with SSS and CIS.

Materials and Methods

Plant materials

Mature leaf segments of *D. communis* were cut from 2 month-in vitro culture plantlets (**Figure 5.1a**) and placed abaxial side down onto semi-solid, half-strength Murashige and Skoog (1962) - MS medium supplemented with 30 g/L sucrose, 0.05 g/L my-o inositol and 0.1 mg/L BA (**Figure 5.1b**). The pH of medium was adjusted to 5.8 and solidified with 2 g/L gelrite before autoclaving for 15 minutes at 121 °C. The cultures were incubated under warm-white LED light (40 µmol/m^2/s intensity) for 12 hours a day at 25±2 °C. One month after culture, adventitious shoots were directly developed from leaf segments and herein called pre-induction leaves which those explants were used as plant materials for further experiment (**Figure 5.1c**).

Semi-solid, continuous immersion and temporary immersion cultures of *Drosera communis*

Full-strength MS medium supplemented with 30 g/L sucrose, 0.1 g/L my-o inositol were used in this experiment. The medium were adjusted pH to 5.8 before sterilizing by autoclave machine at 121 °C for 15 minutes. In semi-solid system (SSS), two pre-induction leaves were cultured in 4 oz glass bottle containing 20 mL gelrite (2 g/L)-solidified medium. For continuous immersion system (CIS), two pre-induction leaves were cultured in 125 mL conical flask containing 20 mL liquid medium and shaken by a rotary shaker at 110 rpm. For temporary immersion system (TIS), twin-bottle TIS were used, twenty pre-induction leaves were cultured in growth vessel and 400 mL culture medium (20 mL/leaves) was filled in medium reservoir of TIS. The medium was pumped to growth vessel every 4 hours and let the leaves be immersed for 5 minutes before pumped out. All culture systems were maintained at 25±2 °C under 12 hours/day warm-white LED lamp with 40 µmol/m^2/s light intensity. After 8 weeks of culture, growth and plumbagin production were examined. Each culture system was completed with three replications of 20 pre-induction leaves.

Growth and biomass examination

After 8 weeks of culture, shoot and root number per explant, clump diameter biomass and size of individual shoot were investigated. Fresh weight was measure after blot drying the clumps with tissue paper while dry weight was measured after drying the clumps at 50 °C in hot air oven for 2 days. Clump diameter was measured using a vernier caliper. Each individual shoots were manually separated from the clump and counted. The diameters of individual shoots from 3 explants were measured from the digital photographs by ImageJ software version 1.52a (Abràmoff, Magalhães, & Ram, 2004).

Plumbagin content analysis

Plumbagin extraction and analysis were performed according to protocol of Wongsa et al. (2018) with some adjustments. Fresh aerial tissues of *D. communis* were dried at 50 °C and ground with a mortar. The fine power (0.5 g) was mixed with 1 mL methanol by vortexing for 1 min then sonicated with ultrasonic water bath

(Sonicor® DSC-120TH, USA) at 35 kHz for 30 min at room temperature. Afterward, the mixture was centrifugated at 8,000 rpm for 2 min prior collecting supernatants to a new tube. The residue was then repeatedly extracted two times. The pooled supernatants were then dried in vacuum oven (LabTech LVO-2030, Korea) at 40 °C. The crude extracts were then reconstituted with 1 mL methanol before filtering through 0.45 µm nylon membrane filter (ANPEL Laboratory Technologies, China). The plumbagin content in the filtered extract was examined by HPLC (Agilent 1100 series, Germany) equipped with a photodiode array detector (Agilent G1362A, Germany). Ten microliter samples were automatically injected onto HPLC system. The separation was accomplished on a Symmetry Shield®RP-8 column (Waters, 150 mm length x 4.6 mm ID, 5 µm particle size). The mobile phase consisted of methanol and 0.1 M aqueous acetic acid in the percentage of 45: 55 (v/v). The isocratic mobile phase was pumped at a flow rate of 0.75 mL/min at room temperature and plumbagin was monitored at 270 nm (**Appendix**, **Figure A4**). The content of plumbagin in each injection was calculated using linearity regression equation obtaining from the standard curve (plotting of peak areas against concentration, **Appendix**, **Figure A5**) of genuine plumbagin (Sigma-Aldrich, USA). All samples were analyzed three times.

Experimental design and data analysis

Complete randomized design was used. The differences of each parameter were statistically compared by one-way ANOVA followed by Duncan's new multiple range test (DMRT).

Results

Growth and biomass of *Drosera communis* in semi-solid, continuous immersion and temporary immersion systems

After 8 weeks of culture, no hyperhydricity tissue was observed in all cultured plant. A hundred percent of pre-induction leaves were survived and successfully proliferated into shoot clump in all tested culture systems, but in different levels of growth and proliferation (**Figure 5.2**). When individual clumps were considered, evidently, TIS promoted better growth and development more than SSS and CIS. The highest fresh (3.40 g) and dry (0.36 g) weight per clump was obtained from TIS,

which was significantly higher than SSS and CIS about 3- and 1.8-time, respectively (**Figure 5.3a-b**). Moreover, the greatest shoot number per clump was also gained from TIS (30.4 shoot per clump) which was approximately 1.6-fold over CIS and 2.6-fold over SSS (**Figure 5.3c**). On the contrary, the lowest number of roots per clump was obtained from TIS (**Figure 5.3d**). For the size of clump, larger clump diameter was obviously presented when the pre-induction leaves were cultured by liquid medium either CIS or TIS which was about 1.7-fold bigger than SSS (**Figure 5.2** and **5.3e**). However, the diameters of individual shoot with in the clump of TIS were smaller than those of CIS (**Figure 5.2** and **5.3f**) about 1.4-time.

In term of growth performances per culture container, the number of shoot, fresh and dry weight per container of explant cultured in TIS was massively higher than those of SSS and CIS (**Table 5.1**). In one container, over 600 shoots were obtained from TIS while only 23 and 38 shoots were obtained from SSS and CIS, respectively (**Table 5.1**). Likewise, the maximal biomass (fresh and dry weight) per container was presented from pre-induction leaves cultured under TIS (68.01 g FW and 7.09 g DW) which was greater than those of SSS and CIS up to around 31 and 18 folds, respectively (**Table 5.1**).

Plumbagin production of *Drosera communis* in semi-solid, continuous immersion and temporary immersion systems

The plumbagin production was analyzed after 8 weeks of culture and represented in **Table 5.2**. The highest plumbagin content was found in plants cultured by CIS (4.91 µg/g DW) followed by SSS (4.22 µg/g DW), which showed no significant difference. However, when plumbagin yield per clump was considered, CIS still provided the highest yield (1.59 µg/clump), but no significant difference with TIS (1.27 µg/clump). On the other hand, SSS provided the lowest plumbagin yield (0.99 µg/clump), however, there was no statistically significant difference with TIS. Interestingly, although the lowest plumbagin content was obtained from TIS (about 2-folds lower than CIS), the highest plumbagin yield that calculated per container (µg/container) was gained from TIS (17.30 µg/container) which was higher than those of SSS and CIS up to approximately 12.2 and 6.4 times, respectively.

Discussions

Effectiveness of bioreactor for mass propagation and biomass production of various medicinal plants has previously been reported (Jesionek et al., 2017; Welander et al., 2014). Especially, advance culture techniques such as TIS have been used in order to increase mass propagation efficiency that is difficult to achieve by conventional propagation methods like semi-solid systems (Moreira et al., 2013; K. Y. Paek et al., 2005). From our results, TIS clearly presented as an appropriate culture system for in vitro large-scale production of *D. communis* plants over SSS and CIS. Biomass and number of shoot obtained from TIS were much higher than those of SSS and CIS in both per explant and per container basis (**Figure 5.3** and **Table 5.1**). Principally, TIS comprising alternative cycles of immersion phase that plant dip in liquid medium for a short period but sufficient for plant's medium uptake and aeration phase that medium is draining and allow plant to get oxygen from gaseous environment (Etienne & Berthouly, 2002). Hence, TIS retains good nutrients uptake of CIS, while keep high gas-exchange environments of SSS (Hahn & Paek, 2005; Mosqueda Frómeta et al., 2017) for cultured plant. Moreover, air renewal occurring inside TIS container during the aeration phase perhaps stimulated photomixotrophic process (Mosqueda Frómeta et al., 2017) and increased photosynthetic rate (Murch, Liu, Romero, & Saxena, 2004) of cultured plant. In addition, the toxin gas (mainly ethylene) that accumulated within culture container was probably eliminated during the aeration phase too (Bello-Bello et al., 2010). As a result, growth and multiplication rate of plant cultured with TIS were improved over SSS and CIS. These results have been found in many plant species including *Rhodiola crenulate* (Zhao et al., 2012), *Crescentia cujete* (Murch et al., 2004), *Chrysanthemum* (Hahn & Paek, 2005), *Lessertia frutescens* (Shaik et al., 2010), *Cattleya walkeriana* (Moreira et al., 2013) and *Gerbera jamesonii* (Mosqueda Frómeta et al., 2017). Nonetheless, the size of individual shoots of *D. communis* cultured by TIS was smaller when compared to those of CIS (**Figure 5.2** and **5.3f**). This result may be due to the excessive of new shoot regeneration leading to inhibit the expansion of individual shoot within a clump. C. Wawrosch (2009) have been reported that increasing the number of shoots decreased shoot length of *Drosera rotundifolia* cultured in semi-solid or liquid medium. Likewise, rosette diameter of *D. intermedia* was increased when the number

of shoot per explant was decreased (Rejthar, Viehmannova, Cepkova, Fernndez, & Milella, 2014). Additionally, although no adverse effect was found in this study, previous studies have been shown some of these issues. In embryonic cultures of cork oak, the hydrodynamic force during TIS operation caused tissue necrosis percentage more than SSS (Pérez et al., 2013). Lower shoot proliferation of TIS in comparison to CIS due to lower nutrient absorption was found in apple root stock cultured (Chakrabarty, Dewir, Hahn, Datta, & Paek, 2006). Therefore, TIS operation conditions should be optimized.

For plumbagin production, SSS and CIS presented higher plumbagin content than TIS when calculated per g DW (**Table 5.2**). It was may be because *D. communis* plant cultured with CIS and SSS were more matured than TIS. The leaves of *D. communis* plant obtained from SSS and CIS was bigger and more expanded (**Figure 5.2**) so probably produced higher plumbagin since the leaf was considered as source of plumbagin accumulation in *Drosera* spp. (Baranyai, Bäcker, Reich, & Lindequist, 2016) and mature leaf can produce and store higher secondary metabolite content (Graham et al., 2010; Towler & Weathers, 2015). The similar result was also found in *Lavandula officinalis* that the plant with higher multiplication rate of TIS presented lower content of rosmarinic acid in comparison to the plant cultured with SSS (Wilken et al., 2005).

The quality of raw material is significantly important in medicinal herb industry; the supply of consistent high quantity and quality of plant material is demanded (Murch et al., 2004). TIS was approved to be more efficient for mass production of *D. communis*, consequently, plumbagin production that calculated per container are superior over SSS and CIS (**Table 5.2**). In addition, TIS uses operation time and labor, especially for subculture to small container lower than SSS and CIS therefor the production cost can be vastly reduced. Moreover, TIS can save the cost of gelling agent used in SSS and electricity power of shaker used in CIS. Therefore, TIS is suitable for large-scale production and the results in this study approved the potential of TIS for providing the plant materials of *D. communis* for industrial scale. However, some operation conditions of TIS like immersion time and frequency need to be optimized.

Conclusions

This study is the first report providing the information on the comparison of biomass production in different culture systems of *D. communis*. TIS provide the highest biomass, shoot number per explant and plumbagin yield per container. The large-scale biomass and plumbagin production of *D. communis* was accomplished by TIS.

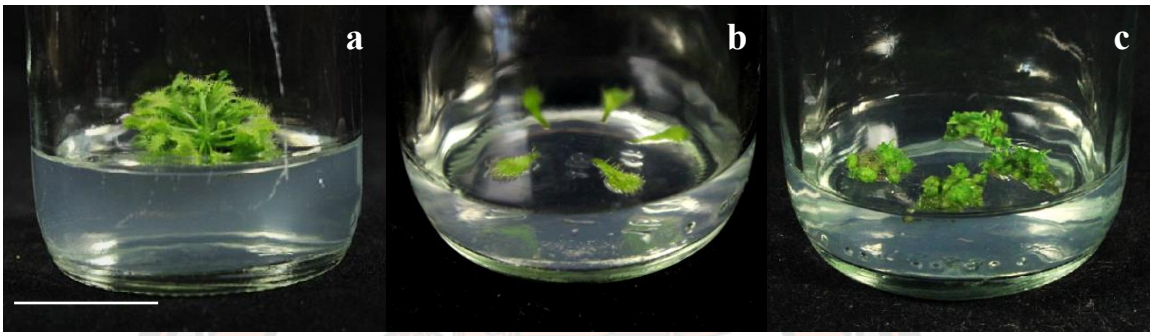

Figure 5.1 Mature leaf segments of *Drosera communis* were cut from 2 month-culture plantlets (a) and placed on half-strength MS semi-solid medium supplemented with 0.1 mg/L BA (b). After one month of culture, leaf segments with regenerated shoots, pre-induction leaves (c) were used as explants in this experiment. Bar = 2 cm.

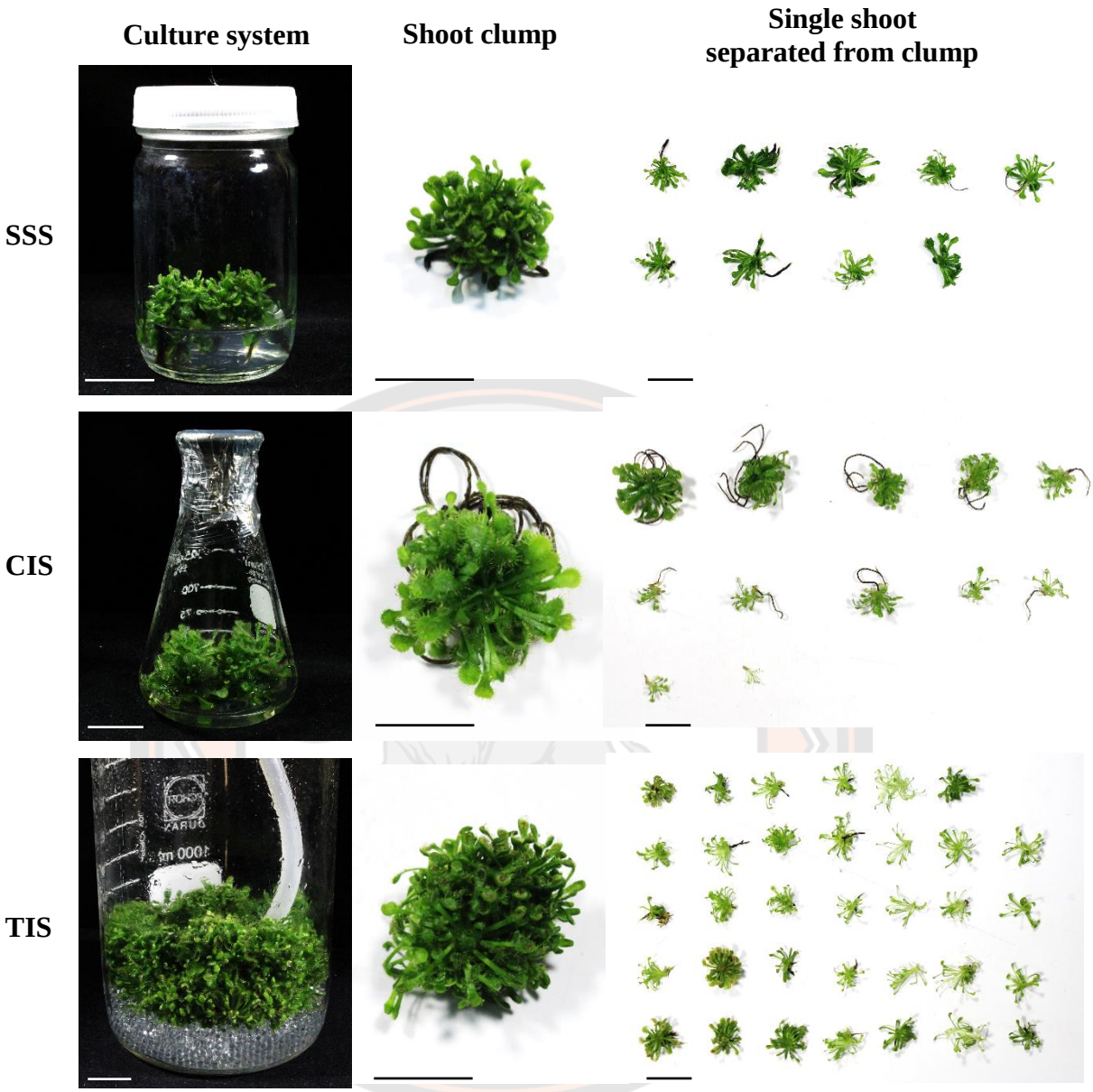

Figure 5.2 Growth and multiple shoot (shoot clump) proliferation from pre-induction leaves (stating explants) of *Drosera communis* after culture with semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) systems for 8 weeks. The medium of TIS was feed every 4 hours for 5 minutes. Bar = 2 cm.

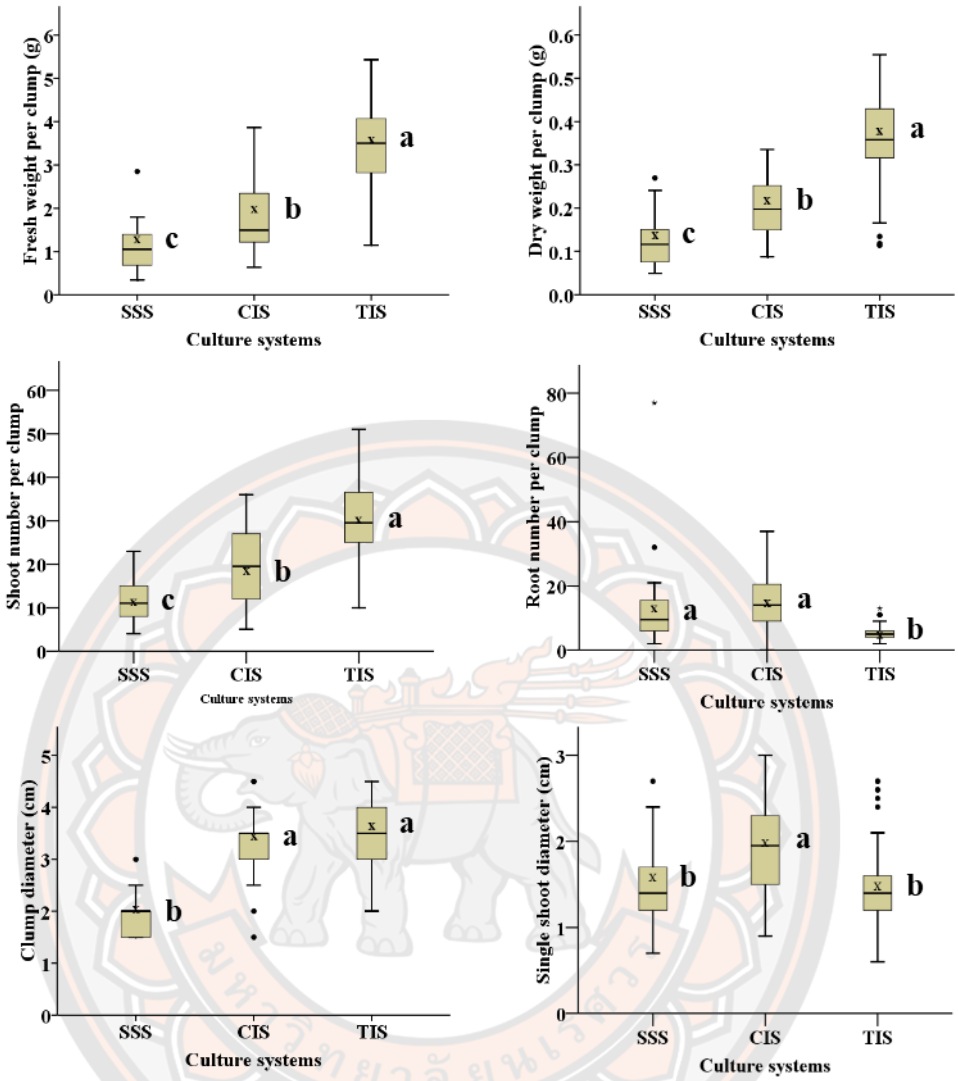

Figure 5.3 Box plots diagram represent fresh weight per clump (a), dry weight per clump (b), shoot number per clump (c), root number per clump (d) clump diameter (e) and single shoot diameter (f) of *Drosera communis* after 8 weeks of culture in semi-solid (SSS), continuous immersion (CIS) and temporary immersion systems (TIS). The bottom and top of the box show the 25th and 75th percentiles, respectively while the bottom and top of whiskers signify the 10th and 90th, respectively. The dot (•) and asterisk (*) represent the outliers and extreme values respectively. The thick horizontal line and the × symbol within the bar are the median and mean, respectively. The different letters beside the bar indicate significant differences of mean at $p \leq 0.05$ according to DMRT. The data of all parameters was obtained from the triplicates of 20 explants per culture system except single shoot diameter was obtained from 30-100 shoots (entire shoots from 3 explants).

Table 5.1 Survival, number of shoot and biomass per container of *Drosera communis* after 8 weeks of culture in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) systems.

Growth parameters	Culture systems		
	SSS	CIS	TIS
Survival rate (%) [1]	100.0 ± 0.0 ns	100.0 ± 0.0	100.0 ± 0.0
Number of shoots per container [2]	23.2 ± 4.5 b	37.6 ± 2.6 b	607.3 ± 47.5 a
Fresh weight per container (g) [2]	2.20 ± 0.81 b	3.63 ± 1.25 b	68.01 ± 6.44 a
Dry weight per container (g) [2]	0.23 ± 0.08 b	0.40 ± 0.09 b	7.09 ± 0.35 a

The same letter within a row is not significantly different at $p \leq 0.05$ according to DMRT. For semi-solid and continuous immersion systems, the first 3 containers showing highest dry weight were selected.

[1] Values are mean ± SD of 3 replicates (20 explants per replicates).

[2] Values are mean ± SD of 3 containers. For semi-solid and continuous immersion systems, the first 3 containers showing highest dry weight were selected.

Table 5.2 Plumbagin production of *Drosera communis* after 8 weeks of culture in semi-solid, continuous immersion and temporary immersion systems.

Culture systems	Plumbagin production		
	Content	Yield	
	(µg/g DW) [1]	µg/clump [2]	µg/container [3]
Semi-solid	4.22 ± 0.98 a	0.99 ± 0.25 b	1.42 ± 0.25 c
Continuous immersion	4.91 ± 0.92 a	1.59 ± 0.32 a	2.70 ± 0.61 b
Temporary immersion	2.44 ± 0.14 b	1.27 ± 0.02 ab	17.30 ± 1.45 a

The same letters within the column are not significantly different at $p \leq 0.01$ according to DMRT.

[1] Values are mean ± SD of 3 individual plant samples.

[2] Values are mean ± SD of 3 clumps that showed the highest weight of each culture system.

[3] Values are mean ± SD of 3 containers. For semi-solid and continuous immersion systems, the first 3 containers showing highest dry weight were selected.

CHAPTER VI

Assessment of Factors Affecting Biomass and Bacoside Productions by *Bacopa monnieri* (L.) Wettst. in Temporary Immersion System

Summary

Cultivation of *Bacopa monnieri* or brahmi as an important medicinal herb in Ayuvedic medicine through temporary immersion system (TIS) were investigated. The explant of *B. monnieri* regenerated shoots from leaf segment was used to demonstrate the effect of immersion frequency, duration and inoculum density on growth and bacoside production of *B. monnieri* through TIS. Thereafter, the best TIS condition was compared to the conventional culture systems. The results revealed that 3 immersion per day for 10 min duration was suitable immersion condition to promote growth and biomass accumulation of *B. monnieri*. Which cultivation with 20 explants per TIS container offered the best inoculum density for large-scale production of *B. monnieri*. However, various concentrations and yields of bacosides were obtained from different TIS conditions. Shortening duration of immersion seem to be improve production of bacosides more than long period of immersion. Whereas, utilization with lowest inoculant density, that provided highest bacoside content but in yield per container was lowest. Furthermore, several statistically significant differences of growth and biomass accumulation were observed in TIS compared to CIS and SSS. In contrast, bacoside A, bacopaside II and bacopasaponin C production in *B. monnieri* obtained from microcontainer was higher than TIS significantly. However, when considered yield found that TIS was shown the obtainable yield maximum.

Introduction

Bacopa monnieri (L.) Wettst. commonly called “brahmi” is an Indian aquatic herb in Plantaginaceae family, it mainly grown and distributed throughout the topical regions of the world in wetlands and muddy shores. The plant is a creeping succulent

herb, glabrous stem having ascending branches, rooting at nodes (Kaur, Nautiyal, & Pant, 2013).

B. monnieri has been widely utilized in traditional system of Indian medicine for centuries as an Ayurvedic medicine. It has been used for almost 3,000 years and is classified as a drug used to enhance memory development, learning and intellect (Majumdar, Basu, Paul, Halder, & Jha, 2013). Recent biotechnological advance, several therapeutic properties of *B. monnieri* have been clinically tested and established. Hence, *B. monnieri* was placed second in a priority list of the most important medicinal plants by National Medicinal Plants Board of India (NMPB) assessed on the basis of commercial value, medicinal importance and potential for further research and development (Haque et al., 2017; Majumdar et al., 2013). The plant has been extensively used as active ingredients in folk and traditional medicine for a nerve, cardio and brain tonics to help or therapeutic agents (RH Singh & Singh, 1980). Besides, it has the potential in many pharmacological effects included central nervous effects; memory enhancement, anti-parkinsonian, anti-oxidant, anti-depressant, anti-ulcer, hepatoprotective, anti-cancer, anti-inflammatory, smooth muscle relaxant, vasodilator and mast cell stabilizer (Al-Snafi, 2013; Sundriyal, Rawat, & Singh, 2013). Additionally, many pharmaceutical and clinical research have concluded that this plant has non-toxic for human consumption (Yadav, Ahmad, Chaudhary, & Ahmad, 2012). A lot of secondary metabolites such as alkaloids, glycosides, flavonoids and triterpenoid saponins were found in *B. monnieri* extraction (Majumdar et al., 2013). Among of them, the major chemical components are composed of steroidal saponins, called bacosides (Pitt & Leung, 2016). The bacosides obtained from *B. monnieri* extract are two forms; bacosides A and B fraction as core active ingredients (Rajbir Singh, Ramakrishna, Bhateria, & Bhatta, 2014; Tripathi, Chouhan, Saini, & Tiwari, 2012). Especially, bacoside A fraction as mixtures of triglycosidic saponins consists of bacoside A3, bacopaside II, bacopaside X and bacopasaponin C, that act as memory improvement agents (Hebert, Weuve, Scherr, & Evans, 2013). In the last few years, the commercial demand of *B. monnieri* was highly consumed by herbal industries around 1,000 tons during 2011 (Muthiah, Shunmugiah, & Manikandan, 2013). Which the requirement of raw materials of this plant is predictable to increase further, which may be due to the popularity of utilization as

memory enhancing drugs. Unfortunately, the natural cultivation of this herb is restricted to grow under conditions of wetlands and muddy shores and dependent on seasonal changes (Sharma, Khajuria, & Mallubhotla, 2013). While propagation of *B. monnieri* through seeds is difficult because of short viability, likewise vegetative propagation by stem are slow process (Rathore & Singh, 2013; Tiwari, Tiwari, & Singh, 2001). In this circumstances, the development of propagating methods for ensuring the obtainability of raw material of a consistent quantity and quality from resources is mandatory.

Plant tissue culture technology has been widely known as an effective tool to propagate several valuable medicinal plants as well as applied in case of *B. monnieri*. Consequently, the in vitro biomass production is alternative opportunity to produce massive amounts of pharmaceutical active compounds round the year without any agricultural land (Haque et al., 2017). Although micropropagation of *B. monnieri* has been studies by many researchers for long time ago. Based on their medicinal properties, the supplementary examination is much desired for further improvement of the micropropagation protocols (Kaur et al., 2013; Mohanta & Sahoo, 2014; Roy, Bihari, & Singh, 2017; Vijayakumar, Vijayakumar, & Stephen, 2010). Nevertheless, establishment of their protocols might be obstructed due to high production cost, more area usage to large production and high cost of labour. With limited commercial use of micropropagation to products with a very high value (Etienne & Berthouly, 2002). To reduce the limitations along with increase efficiency of production during plant propagation, The cultivation methods of plant tissue culture need to develop for scale up systems and automation (Aitken-Christie, 1991).

Bioreactors described according to Mehrotra et al. (2007) are self-container provided optimum growth conditions by regulating chemical or physical parameters to accomplish either maximum yield and high quality of plant or reducing cost of plant production (Preil, 2005). The bioreactors containing liquid media are used for large scale plant propagation along with secondary metabolites production in industrial cultivation. The first utilization of bioreactor was reported for micropropagation of *Begonia* in 1981 (Takayama & Misawa, 1981). Since then it has been developed until the bioreactors has been available to use and success in many horticultural and medicinal plants (K. Y. Paek et al., 2005). However, using liquid

culture systems based on bioreactors have never been totally satisfactory. On the other hand, they were only few successful in some plant species. Actually, these systems developed in the past are not suitable for plant micropropagation, but mainly utilized for microbial cultivation (K. Y. Paek et al., 2005). The advantages of bioreactors for in vitro plant propagation are often counterbalanced. The complete immersion of plant cell, tissue or organ into liquid medium long time frequently causes malformations and loss of plant materials (Debnath, 2011; Teisson & Alvard, 1999). Therefore, to overcome the difficulties, temporary immersion systems (TIS), new design of plant bioreactors for plant cultivation have been established (Harris & Masson, 1983).

TIS has been designed in order to combine the positive effects of liquid and semi-solid culture system, based on restricted contact of the explants with the liquid medium by alternating cycles of immersion periods. TIS keep good nutrients uptake of liquid culture system while retain high gas transfers rate and lower mechanical stress of semi-solid culture system. These two appearances are not usually combined in liquid culture procedures (Berthouly & Etienne, 2005). Recently, various plant species have been accomplished with TIS, for instance coffee (Ducos et al., 2008), orchid (Moreira et al., 2013), potato (Pérez-Alonso et al., 2007), banana (Carlos E Aragón et al., 2005), strawberry (Hanhineva & Karenlampi, 2007), sugar cane (M. d. A. Silva et al., 2015) and eucalyptus (McAlister, Finnie, Watt, & Blakeway, 2005). Different types of TIS have been developed and are widely used in micropropagation of commercial important plant along with adapted for research of secondary metabolite production in medicinal plants (Etienne & Berthouly, 2002; Georgiev et al., 2014). Twin-flasks TIS, one of the initially developed TIS, is simple and flexible operation, so popularly used in several commercial plants (Lyam et al., 2012; Watt, 2012). Generally, the efficiency of TIS is decisively depended on modification of the frequency and duration of medium immersion, that enables a better control of good plant morphology (Etienne & Berthouly, 2002; Mosqueda Frómeta et al., 2017; B. Zhang et al., 2018). The immersion time is very important, since it determines nutrient uptake and control gas ventilation. That influence on growth and development of culturing plant inside of culture container (Pérez et al., 2013). Furthermore, The inoculation density in initial cultivation has been previously reported that it was influenced the success of micropropagation via TIS (J. F. Yang,

Piao, Sun, & Lian, 2010; Young, Murthy, & Yoeup, 2000). However, more increase of starting inoculum lead to reduce the growth rate and increase the necrotic of plant tissue due to the accumulation of growth inhibition factors inside of the culture vessel (Ducos et al., 2008). However, in previous reports, they are very less information about large scale production of *B. monnieri* using temporary immersion system. Therefore, the aims of this research were to investigate the effect of immersion times and inoculum explant density as well as different culture systems on propagation efficiency and bacoside production of *B. monnieri* in in vitro culture.

Materials and Methods

Plant materials

Bacopa monnieri used in this experiments was obtained from sterilized condition (**Figure 6.1a**). the mature leaves (3^{rd} pairs from the top of shoot) were cut and placed onto semi-solid half-strength Murashige and Skoog (MS) culture medium (Murashige and Skoog, 1962) supplemented with 15 g/L sucrose, 0.05 g/L my-o inositol, 0.1 mg/L BA and solidified with 2 g/L agar-gelrite. The pH of culture medium was adjusted to 5.8 before sterilized culture medium by autoclave condition. The explant preparation was maintained in growth root at 25±2 ºC under warm-white LED lamps (40 μmol/m^2/s) for 12 h photoperiod (**Figure 6.1b**). After 15 days of inoculation, shoot buds were regenerated from the cut edges of leaf segments. Which these explants would be used as starting materials for further experiments (**Figure 6.1c**).

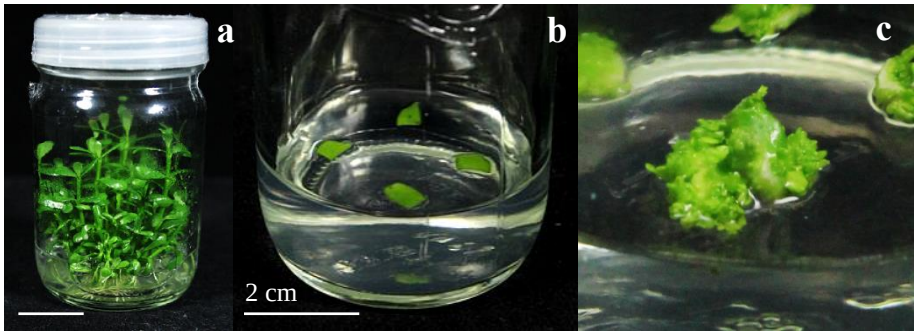

Figure 6.1 Plant materials preparation. Leaf segments of *Bacopa monnieri* were cut from 1 month-culture plantlets (a) and cultured on ½MS medium supplemented with 0.1 mg/L BA (b). After 15 days of culture, leaf segments with regenerated shoot buds (c) were used as plant material in this experiment.

Effect of immersion frequency and duration in temporary immersion system on growth and bacoside productions of *Bacopa monnieri*

The combinations of frequencies and durations of immersion time were used to investigate the propagation efficiency and bacoside production of *B. monnieri* cultured in twin-bottle temporary immersion system. The nine immersion intervals including 3, 6 and 12 times of immersion per day during 1, 5 and 10 min of immersion durations were examined. Twenty explants of *B. monnieri* (**Figure 6.1c**) were placed into plant vessel of TIS and 400 mL of MS culture medium supplemented with 30 g/L of sucrose, 0.1 mg/L of my-o inositol was contained in each medium reservoir. The pH of medium was adjusted to 5.8 before autoclaving at 121 ^{o}C for 20 min. All treatments of TIS were incubated under 12 hour photoperiod from warm white LED lamps at 40 μmol/m^2/s of light intensity. The TIS cultures were maintained in growth room controlling temperature at 25±2 ^{o}C for 4 weeks. Three TIS were incubated per treatment and the experiment was repeated only once. Biomass accumulation and bacoside production of *B. monnieri* were monitored after 4 weeks culture in TIS different immersion frequencies and durations.

Effect of inoculum density in temporary immersion system on growth and bacoside productions of *Bacopa monnieri*

Four inoculum densities comprised with 5, 10, 15 and 20 explants per vessel were performed for growth and bacoside production of *B. monnieri*. The same feature of *B. monnieri* explants (**Figure 6.1c**) were placed in TIS vessel with different initial inoculation. 400 mL of MS culture medium supplemented with 30 g/L of sucrose, 0.1 mg/L of my-o inositol was contained in each medium reservoir. The immersion frequency and duration time was selected from the result in previous the first experiment. All inoculation densities of TIS were kept in growth room controlled temperature at 25±2 ^{o}C and 12 hour of photoperiod using warm white LED at 40 μmol/m^2/s of light intensity for 4 weeks culture. Three replications of each treatment and one repeat of this experiment were carried out. Biomass accumulation and bacoside production of *B. monnieri* were observed and assessed after 4 weeks culture.

Comparison of semi-solid, continuous immersion and temporary immersion cultures on growth and bacoside productions of *Bacopa monnieri*

In order to investigate the effect of different culture systems on propagation efficiency of *B. monnieri* plants for mass production. Semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) systems were compared. The culture medium used in this experiment was full-strength MS culture medium supplemented with 30 g/L of sucrose, 0.1 g/L my-o inositol and pH of medium was adjusted to 5.8 with 1 N NaOH or HCl before sterilized medium by autoclave condition. SSS treatment; two explants of *B. monnieri* (**Figure 6.1c**) were incubated in 4 oz bottle containing 20 mL of semi-solid medium (solidified with 2.0 g/L gelrite). For CIS treatment; the same number and future of explant using in SSS treatment were placed in 125 mL conical flask containing 20 mL of culture medium. The flask mouth was closed with aluminium foil. Thereafter, those flasks were shaken at 110 rpm by rotary shaker. TIS treatment; 1 L of twin-bottle was carried out to use in this experiment. Twenty explants of *B. monnieri* were transferred into plant vessel and contained 400 mL of liquid culture to medium reservoir. This TIS system was determined medium feeding time at 3 times per day in 10 min per time. All of culture systems were maintained in growth room condition at 25±2 ^{o}C under warm white LED lamp (40 μmol/m^2/s light intensity) for 12 hour per day. Each treatment of this

experimental unit had been attempted to three replications; one set of TIS, thirty bottles of SSS and thirty flasks of CIS were accomplished per replicate.

Chlorophyll and carotenoid measurement

To measure the concentration of chlorophyll and carotenoid, weighed fresh leaves (0.02 g) of each treatment were homogenized using a mortar and pestle. 1 mL of cold extraction buffer mixture of 80% acetone and absolute ethanol (1: 1 v/v) was added to crush the leaf, mixed and collected to 1.5 mL tube. Their samples were centrifugated at 10,000 rpm for 5 min and supernatant was divided into quartz cuvattes. The absorbance was measured at 441 nm for carotenoids, 645 nm for chlorophyll b and 663 nm for chlorophyll a. The contents were determined using the following formulas. Where A is absorbance value and LFW is the fresh weight of leaf (g)

$$\text{Chlorophyll a} = [\,(12.25 \times A_{663}) - (2.55 \times A_{645})\,] \times [\,1 / (100 \times \text{LFW})\,]$$

$$\text{Chlorophyll b} = [\,(20.30 \times A_{645}) - (4.91 \times A_{663})\,] \times [\,1 / (100 \times \text{LFW})\,]$$

$$\text{Chlorophyll a+b} = [\,(7.34 \times A_{663}) + (17.76 \times A_{645})\,] \times [\,1 / (100 \times \text{LFW})\,]$$

$$\text{Carotenoid} = [\,((4.46 \times A_{441}) - \text{Chlorophyll a}) + \text{Chlorophyll b}\,] \times [\,1 / (100 \times \text{LFW})\,]$$

Bacoside analysis

The investigation of bacoside A3, bacopaside X, bacopaside II and bacopasaponin C was carried out using the modified method described before (Bansal, Reddy, & Kumar, 2016; Phrompittayarat et al., 2008). Briefly, the aerial parts of *B. monnieri* plants obtained from individual treatment were dried at 45-48 ^{o}C for 2 days and then finely powdered using a mortar and pestle. Samples (0.1 g fine powder) was transferred into 2 mL amber centrifuge tube containing with 1 mL methanol and mixed completely by vortex for 1 min. Thereafter, the mixture was sonicated within an ultrasonic water bath (S50R Elmasonic, Elma, Germany) for 15 minutes and then centrifuged at 8,000 rpm for 3 min. Subsequently, 1 mL of supernatant was collected into new tube and their crudes were re-extracted with 1 mL of methanol for 2 times (round 3rd, supernatant was collected only 0.5 mL and separated to each tube). The extractions were evaporated under dark condition at 40

^{o}C until dry (about 36 h). Afterward, the residues were reconstituted with 0.5 mL of methanol in each tube to redissolve, finely vortexed for 2 min, spin down and pooled it together. The final volume was adjusted to 1 mL and filtrated through a 0.45 µm nylon syringe filter (Tianjin Fuji Science & Technology Co., Ltd., China) before kept into 1.5 mL vial. The extractions were maintained under -80 ^{o}C until required for quantification using high performance liquid chromatography (HPLC).

The extracted samples were analyzed by high performance liquid chromatography with diode array detection (HPLC-DAD) (Agilent 1100 series, Germany). Separation was accomplished in a Purospher® STAR RP-18 column (250 x 4.6 mm, 5µm particle size). The mobile phase consisted of 0.2% aqueous phosphoric acid and acetonitrile (65: 35 v/v). The flow rate was adjusted to 1.0 mL/min for 60 min saturation time. The bacosides content were detected at 205 nm and the content of bacosides was calculated by comparing relative retention times with standard samples.

Experimental design and data analysis

Complete randomized design was used in all experimentations. The significant difference of each parameter was statistically compared by one-way ANOVA followed by Duncan's new Multiple Range Test (DMRT) at $p \leq 0.05$.

Results

Effect of immersion frequency and duration on growth and bacoside productions of *Bacopa monnieri* in temporary immersion system

In order to investigate and determine an optimal immersion condition for growth and multiplication of *B. monnieri* culturing with TIS, the different immersion frequencies and durations were performed for 4 weeks culture. Leaf segments of *B. monnieri* were different growth and shoot multiplication in among TIS, combining different feeding of culture medium between three immersion frequencies and three immersion durations (**Figure 6.2**). The results revealed that a hundred percentage of survival rate was found in all TIS treatments. Except using 12 immersions per day with 10 min per time, that showed slight decreasing plant survive (95%) (**Table 6.1**).

During cultivation of *B. monnieri* by different immersion times, growth quality of shoot clump and plantlets abnormality were also exhibited differently (**Figure 6.3)**. Severe leaf necrosis of *B. monnieri* was observed more than 60%, cultured with high level of immersion frequency (12 times per day) and would be increased in higher than 80%, when 5-10 min of immersion durations was used (**Figure 6.3b** and **Table 6.1**). Contrary with using 6 times per day for 5 min per time, that was only one TIS condition showed no severe leaf necrosis (**Table 6.1**). Furthermore, Severe leaf chlorosis of *B. monnieri* could be found more than 46 % in all of immersion treatments and up to 97% using in highest of immersion times (12 times per day in 10 min per time) (**Figure 6.3c** and **Table 6.1**). While, hyperhydric symptom of *B. monnieri*, occurred at the base of *B. monnieri* clump (**Figure 6.3c**), were lower appeared than 20% in all of immersion treatments. Except using 12 times per day for 10 min per time, that induced hyperhydric symptom of plantlet more than 50% (**Table 6.1**).

Besides, these results indicated that proliferation and elongation of *B. monnieri* shoots were obtainable to be affected by frequency and duration of immersion. Which using of 12 time per day for 10 min per time gave a maximum the number of shoots (31.6 shoots per clump). In contrast with shortening immersion interval and period (3 times per day for 1 min per time) resulted in lowest shoot numbers (18.7 shoots per clump). Whereas, the extension of frequency or duration led to improve shoot production of *B. monnieri* in TIS (**Figure 6.4a**). Shoot elongation and node number of *B. monnieri* were same trend of establishment. Using 3 immersions per day provided the highest shoot elongation and node number of *B. monnieri* more than 10 cm and 7 nodes per shoot, respectively (**Figure 6.4b**, **c**). Whereas, declining of those growth parameters was observed after immersion frequency and duration increasing (**Figure 6.2**).

Biomass accumulation of *B. monnieri* cultured in TIS. The maximum clump fresh weight of *B. monnieri* was found in 6 times per day for 1 min per time (2.93 g per clump) and 12 times per day for 10 min per time (2.94 g per clump). While, increasing the immersion duration could enhance clump fresh weight especially using with 3 or 12 times per day, contrary using with 6 times per day the weight of clump was decreased (**Table 6.2**). In terms of fresh and dry weight of aerial part, that they

was similarly results in clump fresh weight. However, when comparison the total dry weight of each culture container obtained from different immersion treatments. No significant differences were recorded from total dry weight per TIS container. Except using with 12 immersions per day for 1 min per time showed lowest obtainable dry weight per container (1.54 g per container) (**Table 6.2**).

Photosynthetic pigments, chlorophyll a, b, a+b and carotenoid, obtained from leaf of *B. monnieri* were investigated after culture in different immersion conditions. The results revealed that the immersion frequency and duration had effect on content of chlorophyll and carotenoid in their leaves (**Figure 6.5**). Which immersion interval seem to influence more than immersion duration. Whereas, increasing immersion frequency caused to decrease chlorophyll and carotenoid contents. When consideration of immersion frequency at the same immersion duration, particular 1 and 10 min per time. The results showed that the immersion of 3 and 6 times per day significantly presented content of all pigments higher than using with 12 times per day (**Figure 6.5**).

Four bacoside compounds, consist of bacoside A, bacopaside II, bacopaside X and bacopasaponin C obtained from *B. monnieri* in TIS were investigated by HPLC techniques. Which the amount of those compounds varied depending on the immersion frequency and duration (**Figure 6.6**). From the results, shortening period of immersion (1 min per time) seem to be the optimal duration time of TIS to provide all of bacosides in high content level. Whereas, using long-time of duration (10 min per time) gave lower accumulation of bacoside content than other treatments (**Figure 6.6**). Especially, all of bacosides were showed lowest content in 12 time per day for 10 min per time (0.27%DW of bacoside A, 0.43%DW of bacopaside II, 0.21%DW of bacopaside X and 0.64%DW of bacopasaponin C) (**Figure 6.6**). Moreover, when consideration of the total yield of bacosides gained from different treatments, that the results were showed the same trend with percentage bacoside content value. Using 3 immersion time per day, the modifications of immersion duration were almost no effect on yield of bacoside production, unlike with increasing of immersion frequency (6 and 12 times per day) (**Figure 6.6**). In overall immersion treatments, the lowest of bacoside yield was found in 12 times per day for 10 min per time (**Figure 6.6**).

Effect of inoculum density on growth and bacoside productions of *Bacopa monnieri* in temporary immersion system

The inoculation density is another one of physical factors influencing on growth and morphology during cultivation with TIS. The results revealed that the different inoculant amounts of explants within TIS were obviously effect on growth and morphology of *B. monnieri* (**Figure 6.7**). A hundred percentage of survival rate was found in all inoculum densities. Although 20 explants per container as highest density using displayed a little appearance of severe leaf necrosis and hyperhydric plantlet (1.7% and 1.7%, respectively), but not significant different between using the lower density (**Figure 6.3b, d** and **Table 6.3**). Whereas, the highest severe symptom of leaf chlorosis was found in TIS containing 5 and 30 explants per container (73.3% and 80.0% respectively) (**Figure 6.3c** and **Table 6.3**). In term of shoot multiplication of *B. monnieri* cultured with different inoculant densities. After 4 weeks, the results revealed that an optimal density of 20 explants was identified in TIS. Plantlets presented the greatest shoot numbers (27.9 shoots per clump), but not significant different from using 5 or 15 explants per container (26.7 and 24.6 shoots, respectively). While the lowest of shoot production showed in 10 explants per container (22.3 shoots per clump) (**Figure 6.8a**). Furthermore, using 5 explants per container as low inoculant density, *B. monnieri* plantlets substantially showed lowest shoot elongation (6.9 cm per shoot). Whereas, increasing number of culturing explants in container, shoot height of *B. monnieri* had rapid increasing trend. Which shoot height was highest in 20 explants per container (12.7 cm per shoot) (**Figure 6.8b**). Likewise, shorter shoots of *B. monnieri* obtained from 5 inoculant density were also provided lowest of node numbers (6.6 nodes per shoot) (**Figure 6.8c**). Subsequently, biomass accumulation of *B. monnieri* plantlets obtained from different inoculum was investigated; weight of individual clump, aerial part of fresh and dry weight and total obtainable dry weight per TIS container. The maximum clump fresh weight of *B. monnieri* was harvested from inoculant density of 20 explants (2.92 g per clump) and 5 explants (2.80 g per clump) (**Table 6.4**). Although the highest of fresh weight of aerial part was found in using with 20 explants per container (2.14 g per clump), but all treatments of density were no significant difference of aerial part dry weight (**Table 6.4**). In addition, the greatest amount of total dry weight (3.01 g per

container) were represented in 20 explants of inoculum density and would be decreased according to lower inoculum density (**Table 6.4**).

Nevertheless, the estimation of photosynthetic pigments in their leaf of *B.* monnieri in different inoculum density was performed to investigate chlorophyll a, b, a+b and carotenoid contents. The values of those pigments were shown no significant differences at $p \leq 0.05$ (**Figure 6.9**). Following investigation of bacosides accumulation in *B. monnieri* cultured in various density, the results indicated that there was linear correlation between amounts of inoculum explant and bacoside A3 and bacopaside X content (**Figure 6.10**). The substantial highest concentration of bacoside A3 and bacopaside X was observed in TIS containing 5 explants per container (0.83%DW and 0.38%DW, respectively) and be gradually decreased when increasing inoculum density (**Figure 6.10**). Whereas, lowest content of bacopaside II showed in density of 15 explants (0.61%DW), contrary to other densities that provided higher than 0.8%DW of bacopaside II (**Figure 6.10**). However, only *B. monnieri* cultured in TIS with 20 explants per container, that represented higher accumulation of bacopasaponin C than other inoculum densities, significantly. Moreover, when comparison of available yield between different starting explants in TIS. The results obviously suggested that 20 explants per container of TIS was appropriated density providing highest yield of bacosides (11.8 mg of bacoside A3, 24.5 mg of bacopaside II, 5.7 mg of bacopaside X and 34.32 mg of bacopasaponin C) (**Figure 6.10**).

Comparison of semi-solid, continuous immersion and temporary immersion cultures on growth and bacoside productions of *Bacopa monnieri*

In vitro comparison of *B. monnieri* cultivation with among the three different systems exhibited different features as observed (**Figure 6.11**). Several statistically significant differences were observed in growth production and biomass accumulation. The highest number of shoots of *B. monnieri* was found in TIS treatment (27.9 shoots per clump), which more than nearby 2 folds in CIS (14.5 shoots per clump) and SSS (12.9 shoots per clump) (**Figure 6.12a**). Whereas, TIS could also induce elongation of shoot higher than 12 cm and 7 nodes per shoot (**Figure 6.12b, c**). In contrast using with SSS, that this system served all grown

parameters of *B. monnieri* in lowest (12.9 shoots per clump, 4.6 cm of shoot height and 4.4 nodes per shoot) (**Figure 6.12**). In addition, the greatest biomass accumulation of *B. monnieri* was significantly indicated in TIS treatment, including clump fresh weight (2.74 g per clump), which higher than around 2 folds when compared with CIS (1.34 g per clump) and SSS (1.16 g per clump), aerial part of fresh and dry weight obtained (2.14 g fresh weight and 0.14 g dry weight) as well as total dry weight (3.01 g per container), that they were also obtained from TIS treatment after 4 weeks culture (**Table 6.5**).

Chlorophyll and carotenoid investigation, leaf of *B. monnieri* obtained from different systems was measured and indicated that TIS treatments showed a maximum the content of photosynthetic pigments including chlorophyll a, b and a+b more than other culture systems. Whereas, value of carotenoid content was not significant difference (**Figure 6.13**). Furthermore, all of bacosides obtained from TIS was significant represented lower content (%DW) than CIS and SSS, except bacopaside X showed higher (**Figure 6.14**). However, a higher yield of bacosides (mg per container) clearly obtained in TIS than around 6 folds when compared with a CIS and SSS container (**Figure 6.14**).

Discussions

Temporary immersion system (TIS), as one alternative method of plant bioreactor in plant tissue culture, has been successfully used for large-scale production of horticultural crops (K. Y. Paek et al., 2005). Since, immersion time of frequency and duration is the most decisive point of important parameters that affecting the efficiency of temporary immersion systems as well as inoculum density remarkably influence on multiplication rate and obtainable yield (Alvard, Cote, & Teisson, 1993; Etienne & Berthouly, 2002; Georgiev et al., 2014). Consequently, many researchers, utilizing TIS for plant production, have attempted to establish the optimizing protocol for mass propagation and secondary metabolites production in various plant cultivation (Godoy et al., 2017; Pérez et al., 2013; Ramos-Castellá et al., 2014; B. Zhang et al., 2018). Particularly, medicinal plants for instance, *Stevia rebaudiana* (R. Sreedhar et al., 2008), *Digitalis purpurea* (Pérez-Alonso et al., 2009),

Lessertia frutescens (Shaik et al., 2010), *Eurycoma longifolia* (R. Ibrahim, 2017), *Rhododendron tomentosum* (Jesionek et al., 2017).

Effect of immersion frequency and duration in temporary immersion system on growth and bacoside productions of *Bacopa monnieri*

Among the immersion times of frequencies and durations used in temporary immersion system had a little effect on survive rate of *B. monnieri*, while plantlets cultured under high frequency and longtime immerse in liquid medium showed more undesirable characters, a severe leaf necrosis, leaf chlorosis and hyperhydricity. On the other hand, the results indicated that *B. monnieri* plantlets shown the best growth in the less frequency of immersion (3 times per day) with extending duration of immersion (10 min per time), that seem to be more effective condition for biomass accumulation than the other feeding conditions.

This reason might be due to high frequency and longtime duration of immersion involved composition of internal environment, such as humidity and force of liquid motivation in culture vessel, that possibly induce physiological disorders (Dewir et al., 2006; Teisson et al., 1996). Previous research indicated that the levels of hyperhydricity of gerbera shoots were increased, when the explants was highly submerged into the liquid medium (Mosqueda Frómeta et al., 2017). Likewise, many researchers demonstrated that higher immersion frequency (every 2 hours) not only improved biomass accumulation of *B. striata* pseudobulb, but also leads to stimulate hyperhydricity and less vigorous plantlets (B. Zhang et al., 2018). In addition, Martre, Lacan, Just, and Teisson (2001) reported about the effect of immersion frequency on oxidative stress in *Hevea brasiliensis* callus culture, that higher immersion of the explants induced a substantial oxidative stress.

As well know, the frequency and duration of immersions affects to absorption of nutrient and also relates with gaseous renewal of internal culture container (Georgiev et al., 2014; Teisson et al., 1996). Pérez-Alonso et al. (2009) used TIS for encouragement of biomass and secondary metabolite production in some medicinal plants. The results found that fresh and dry weight as well as cardiotonic glycoside content were variously obtained from *Digitalis purpurea* cultured under different immersion frequencies (maximum harvest in 6 immersion per day). Similarly, use of

high immersion frequency (every 6 hour) affect to decrease length of *Gerbera jamesonii* shoot and increase hyperhydricity of stems and leaves (Mosqueda Frómeta et al., 2017). However, large-scale propagation of *Vanilla planifolia* using a TIS, that length of their shoots was favorably increased after incubation in shorter duration times (2 min) with a high frequency (every 4 h) which it does not require prolonged immersion times (Ramos-Castellá et al., 2014). Contrary to *B. monnieri*, the greatest length of shoot was presented in TIS used with less frequency and long duration of immersion (3 times per day and 10 min per time). Nevertheless, the suitability of culture medium feeding for improvement of TIS efficiency may be also depended on plant species and specific organs within same species (Akdemir et al., 2014; Gutiérrez, López-Franco, & Morales-Pinzón, 2016).

HPLC analysis revealed that concentration and yield of all bacosides were differently obtained from growing *B. monnieri*. Which the option allowed the improvement of accumulation of bacosides by changing frequencies and durations of immersion. Short immersion interval, both frequency and duration, looked like appropriate feeding condition to increase those productions. This probably because utilization of suitable immersion interval may be not obstructed to phytocompound accumulation (Pérez-Alonso et al., 2009). Whereas, many researchers described the positive relationship between biomass accumulation and secondary metabolite production (Roca-Pérez, Boluda, Gavidia, & Pérez-Bermúdez, 2004). Although, these results displayed that the highest of aerial part dry weight (g/clump) and total dry weight (g/container) of *B. monnieri* cultivation was observed from 12 immersions per day with 10 min per time, but available contents (%DW) and yield (mg/container) of all bacosides was lowest. Which the inverse relation between biomass and interesting compounds has been reported by some researchers (Wilken et al., 2005). Till date, several reports have been published the influence of immersion times on secondary metabolite production in some valuable plants (George et al., 2008; Pérez-Alonso et al., 2009; Quiala et al., 2006). Georgiev, Ivanov, Berkov, and Pavlov (2013) investigated the alkaloid production of *Pancratium maritimum* L. from shoot culture by TIS, every 12 hour of immersion interval with 15 min duration, shoot could accumulate maximal amounts of biomass and also provide higher concentration of alkaloid compounds than using with more or less immersion times. However, the

influences of these factors on plant growth and bioactive compounds seem to depend on specific plant species and culture methods (R. Ibrahim, 2017).

Effect of inoculum density in temporary immersion system on growth and bacoside productions of *Bacopa monnieri*

In order to investigate the optimal inoculum density of *B. monnieri* cultured by TIS, the most suitability of frequency and duration of immersion from previous experiment should be used to determine. Although the bacosides are almost produced from aerial parts of *B. monnieri* such as stems and leaves (Sharma et al., 2013). It is necessary to combine the plant quality, biomass as well bacoside production for decision to determine using the optimal immersion interval. Based on previous experiment, the immersion of 3 immersions per day with 10 minutes duration was chosen to use as the best immersion condition.

The different inoculum densities of TIS did not affect leaf necrosis and leaf hyperhydricity of *B. monnieri*, which may be because utilizing suitable immersion frequency and duration from previous experiment prevented the appearance of undesirable morphological characters. However, leaf chlorosis was also remained exhibition in severe level. Piao, Chakrabarty, Hahn, and Paek (2003) suggested that the necessity to optimize the number of explants per culture container in order to ensure the most effectiveness growth and propagation by TIS, conversely using high inoculant density may cause rapid phenotypic malformations and decrease the plant quality. In fact, a large number of explants within small containers, growth and development of plant might be limited due to restrict some essential factors such as oxygen diffusion, competition of nutrient absorption and lighting (Ducos et al., 2008; B. Zhang et al., 2018). Moreover, toxic gases were increasingly accumulated during the culture within culture vessel, i.e. ethylene. That may induce physiological disorders and/or irregular secondary metabolite production in plant cells or tissues (Roels et al., 2006; Santana-Buzzy et al., 2005). Which these negative things may possibly be occurred in plant tissue culture. The results suggested that maximum growth and biomass accumulation of *B. monnieri* was obtained from 20 explants as highest inoculation density test. Which, this density did not inhibit biomass accumulation, contrarily shown to be effective in obtaining large number of plant

materials. Likewise, the patterns have been reported by Hahn and Paek (2005), they discovered that shoot length of *Chrysanthemum* could be increased with increasing a numbers of single nodes in TIS vessel, while diameters and branches of their stems were obviously reduced after a smaller number of single nodes were used. Furthermore, the similar effect of inoculation density was occurred in the improvement production of potato by TIS (Rahman, Islam, Chowdhury, & Subramaniam, 2015). Whereas, some researchers indicated that using a higher number of explants cause to reduce the photosynthesis and shoot proliferation (Niu & Kozai, 1997). However, it is known that inadequate inoculant had poor potential for large-scale micropropagation by TIS (Hahn & Paek, 2005). Therefore, to evaluate this factor, the TIS experiments should be taken a maximum advantage of capacity of the culture vessels.

Besides, changing the inoculation density of TIS did not affected to chlorophyll and carotenoid contents in leaf of *B. Monnieri* but different from morphology, that they were presented significant differences. Which, using the various immersion times gave more influence on production of photosynthetic pigments than different inoculant density. In addition, the content and yield of bacoside obtained from their plants were also various observations. From the results indicated that reducing the number of *B. monnieri* explants per container seem to improve some of bacoside accumulation (%DW) more than using a lot of density. Whereas, the maximum of all bacosides yields (mg/container) was displayed in highest inoculum density. Although the biomass of explants is usually reported as a part of description of protocol used, only few publication papers have been reported the optimized of initial explants on biomass accumulation and secondary metabolite production with TIS (Piao et al., 2003; Quiala et al., 2014; Schumann et al., 2012; J. F. Yang et al., 2010). Finally, the optional immersion time (frequency and duration) as well as inoculant density may be a useful opportunity for the establishment of large-scale *B. monnieri* production by temporary immersion system.

Comparison of semi-solid, continuous immersion and temporary immersion cultures on growth and bacoside productions of *Bacopa monnieri*

The investigation of culture systems influence on in vitro propagation efficiency of *B. monnieri* was accomplished and compared between semi-solid system and liquid systems including continuous immersion and temporary immersion system. In the most cases, large-scale production of medicinal plant using with microcontainers has not become cost-effective culture systems, since mass production with bioreactors is expensive (Bhojwani & Razdan, 1996). Subsequently, various types of bioreactor including temporary immersion systems have been developed by many researchers to establish to be low-cost culture systems (Jesionek et al., 2017; Steingroewer et al., 2013). The results of these experiments shown an optimized culture system for large-scale production of *B. monnieri* in term of increasing growth and biomass accumulation. Plantlets obtained from temporary immersion system (TIS) showed the better shoot number, shoot elongation and aerial part weight than those obtained in conventional culture systems.

Temporary immersion systems for micropropagation have been exhibited to be beneficial in various plant cultivation. A possible reason for this may the occasional contact between the liquid medium and explants, which periodically supplies the essential nutrients and oxygen transfer, reduce hydrodynamic forces and reduce toxic gases accumulation, improvement of plant quality (Etienne & Berthouly, 2002; Shaik et al., 2010; Vives et al., 2017). Whereas, R. Sreedhar et al. (2008) revealed that shoot of *Stevia rebaudiana* obtained from TIS could elongate higher than in shake flask at the same cultivation condition. Likewise, the micropropagation and bioreactor studies of medicinally important plant (*Lessertia frutescens* L.) described that the best shoot multiplication and growth were performed by temporary immersion culture system significant than continuous immersion and semi-solid culture (Shaik et al., 2010). While, the semi-solid and continuous immersion systems with microcontainers might obstruct shoot proliferation and elongation of *B. monnieri*. Maritza Escalona, Samson, Borroto, and Desjardins (2003) indicated that the periodic renovation of internal gases into culture vessel and intermittent contact of explants with liquid medium in TIS resulted in plants enhancing their association photosynthesis with transpiration. Under ventilation, CO_2 concentration gets higher in

container during TIS operation (Hahn & Paek, 2005; K.-Y. Paek, Hahn, & Son, 2001). Arencibia, Vergara, Quiroz, Carrasco, Bravo, et al. (2013) reported that enrichment of CO_2 inside of culture container could improve plant physiological processes, in this case by photomixotrophic metabolite. Zhao et al. (2012) working with medicinal plant (*Rhodiola cremulata*) related that forced ventilation was employed in the temporary immersion bioreactor in order to decrease the morphological disorders and improve shoot quality and enhance the rate of multiplication. Carlos Eduardo Aragón et al. (2010) and Jova, Kosky, and Cuellar (2011) confirmed that a greater photosynthetic rate and the best growth of plantlets were obtained from TIS when compared with continuous immersion. Which the most important reasons of the TIS efficiency were reported that TIS combine both advantages of gelling medium and liquid medium improved the growth of plantlets (Etienne & Berthouly, 2002; Georgiev et al., 2014). Moreover, the force aeration provide higher gas exchange leading to increase photosynthetic pigments when compared to the closed system without a gaseous renewal (Saldanha et al., 2012).

Although, the production of *B. monnieri* under TIS condition showed greatest growth and biomass accumulation. But in term of bacoside production, the concentration of their bioactive metabolites seemed to be inconsistent with biomass accumulation. In contrast to use with microcontainer such as SSS and CIS, that they provided higher content of bacoside compounds than TIS. Which, the better growth of plant within in vitro condition may be not refer to appropriate accumulation of some secondary metabolites (Grzegorczyk-Karolak, Rytczak, Bielecki, & Wysokińska, 2017; Jesionek et al., 2017). A similar trend was observed in shoot of *Lavandula officinalis*, that the increase of multiplication rate was higher in TIS than culturing with microcontainer. Nonetheless, content of rosmarinic acid, essential compound in this plant showed lower than 10 times comparing with small container (Wilken et al., 2005). However, the different yield of bacosides was clearly observed between TIS and conventional systems. From this result, application of TIS seems to be more profitable for conducting the large-scale of secondary metabolite production. Moreover, TIS is easy to handle and occupy little space in a growth room. There are no reports which refer to comparison of different culture systems on growth and

bacoside production in *B. monnieri*. So, this is the first report for evaluation of *B. monnieri* in various type of culture systems.

The principle prerequisite for the development of medicinal productions is a consistent source and large-scale propagation of plant material. The development of culture system for mass production represents a significant improvement for medicinal products of this species. In addition, this studies of temporary immersion system of *B. monnieri* further support previous researches that improve propagation efficiency of medicinal plants with in vitro mass production by TIS (Affreen, 2008; Murch et al., 2004; Shaik et al., 2010; R. Sreedhar et al., 2008).

Conclusions

This research demonstrated that large sale propagation and secondary metabolite production of *Bacopa monnieri* was accomplished by temporary immersion system. The experimental procedure described that 3 immersion time per day with 10 min using 20 initial explants was the appropriate TIS condition for growth and multiplication of *B. monnieri* in in vitro culture. Although TIS showed some of interesting bioactive compounds lower than culturing with microcontainer. However, the utilization of TIS can be produced a large quantities using very little space. In future, this TIS protocol can be applied to commercially implement by pharmaceutical industries to progressive market demand of *B. monnieri*.

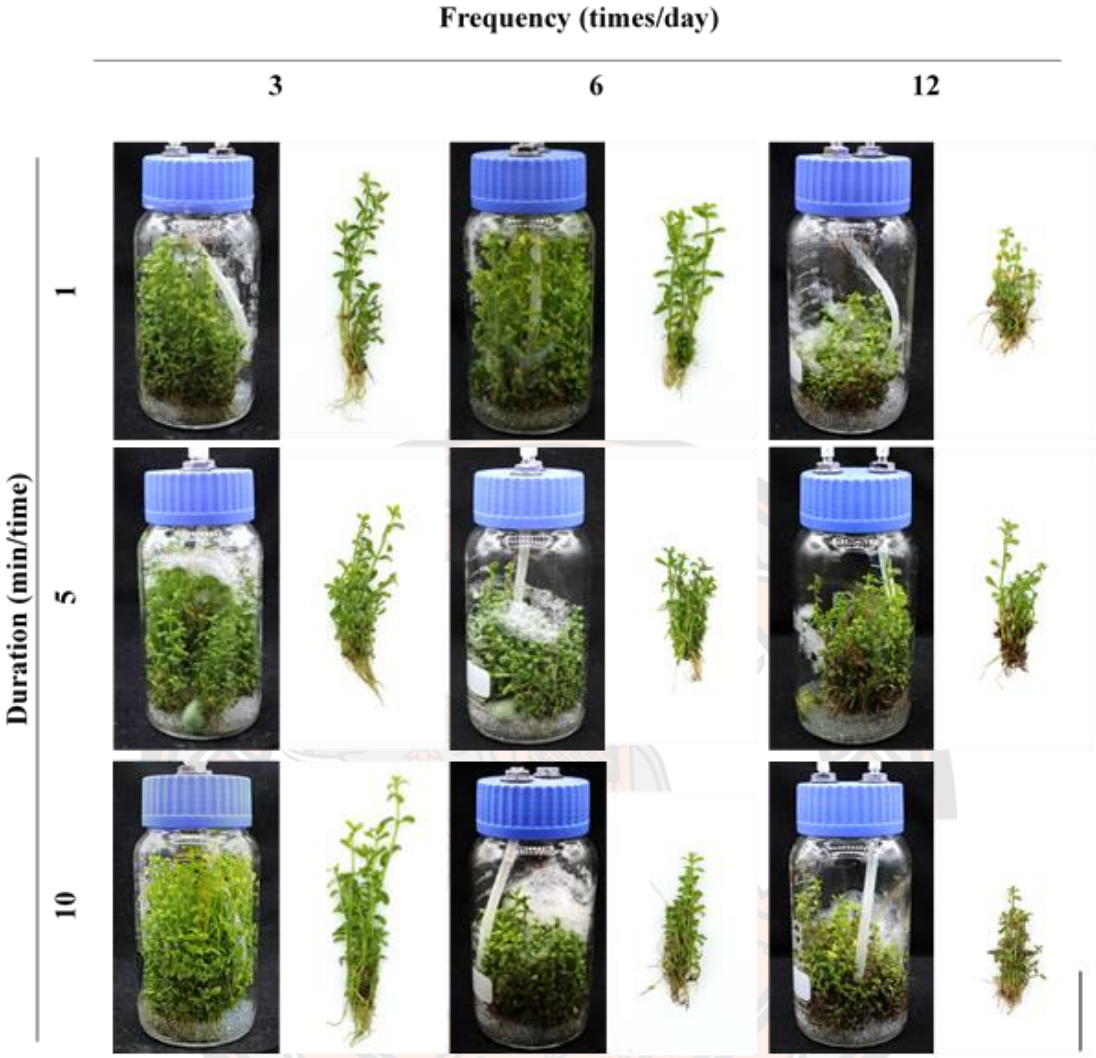

Figure 6.2 Comparative growth and development of leaf segments with regenerated shoot buds of *Bacopa monnieri* after 4 weeks cultivation in temporary immersion system with different immersion frequencies and durations. Bar = 5 cm.

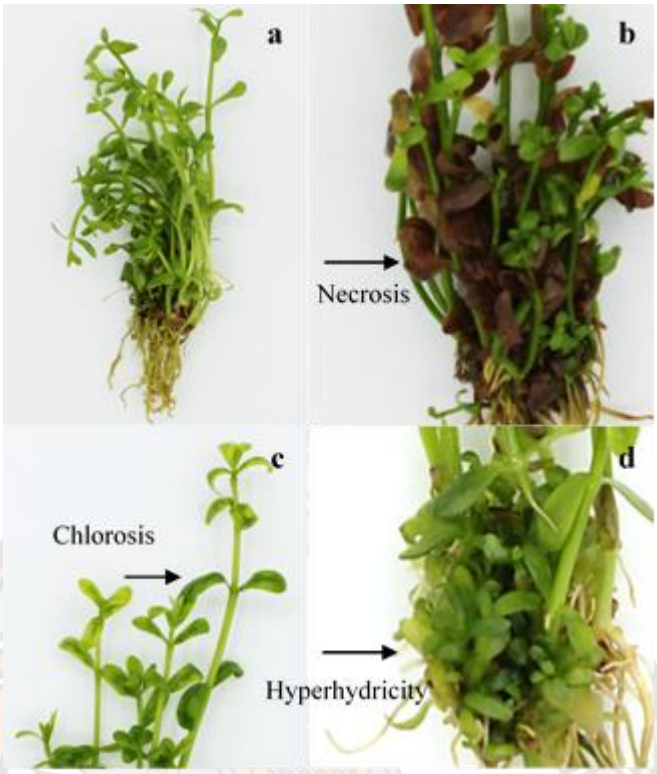

Figure 6.3 Growth quality of *Bacopa monnieri* shoot clump appeared after cultivation in different immersion frequencies and durations of temporary immersion system. Shoot clump with healthy plantlets (a), severe leaf necrosis (b), severe leaf chlorosis (c) and hyperhydricity tissues (d).

Table 6.1 Effect of immersion frequency and duration of temporary immersion system on survival and abnormality rates of *Bacopa monnieri*. The data was recorded at 4 weeks after culture.

Frequency (times/day)	Duration (min/time)	Survival (%)	Severe leaf necrosis (%) [1]	Severe leaf chlorosis (%) [1]	Hyperhydricity (%) [2]
3	1	100.0 ± 0.0 ns	31.1 ± 2.0 cd	55.6 ± 7.0 b	2.2 ± 1.0 b
	5	100.0 ± 0.0	8.9 ± 2.6 cde	62.2 ± 3.6 b	4.4 ± 2.0 b
	10	100.0 ± 0.0	6.7 ± 0.0 de	57.8 ± 6.0 b	0.0 ± 0.0 b
6	1	100.0 ± 0.0	35.6 ± 8.7 bc	55.6 ± 7.2 b	16.0 ± 5.7 ab
	5	100.0 ± 0.0	0.0 ± 0.0 e	46.7 ± 10.7 b	0.0 ± 0.0 b
	10	100.0 ± 0.0	17.8 ± 5.0 cde	46.7 ± 1.7 b	0.0 ± 0.0 b
12	1	100.0 ± 0.0	60.0 ± 3.0 b	48.9 ± 1.0 b	1.0 ± 0.0 b
	5	100.0 ± 0.0	91.1 ± 2.6 a	55.6 ± 6.0 b	20.0 ± 8.9 ab
	10	95.5 ± 7.7	88.5 ± 3.5 a	97.4 ± 1.1 a	54.4 ± 10.4 a

Values are mean ± SE of 3 replicates (20 explants per replicate), except survival are mean ± SD. The same letters within the column are not significantly different at $p \leq 0.05$ according to DMRT, ns; non-significant difference.

[1] Severe leaf necrosis and leaf chlorosis were recorded when plantlet within clump show severe symptoms (**Figure 6.3 b, c**).

[2] Hyperhydricity was observed from leaves at the base of clump.

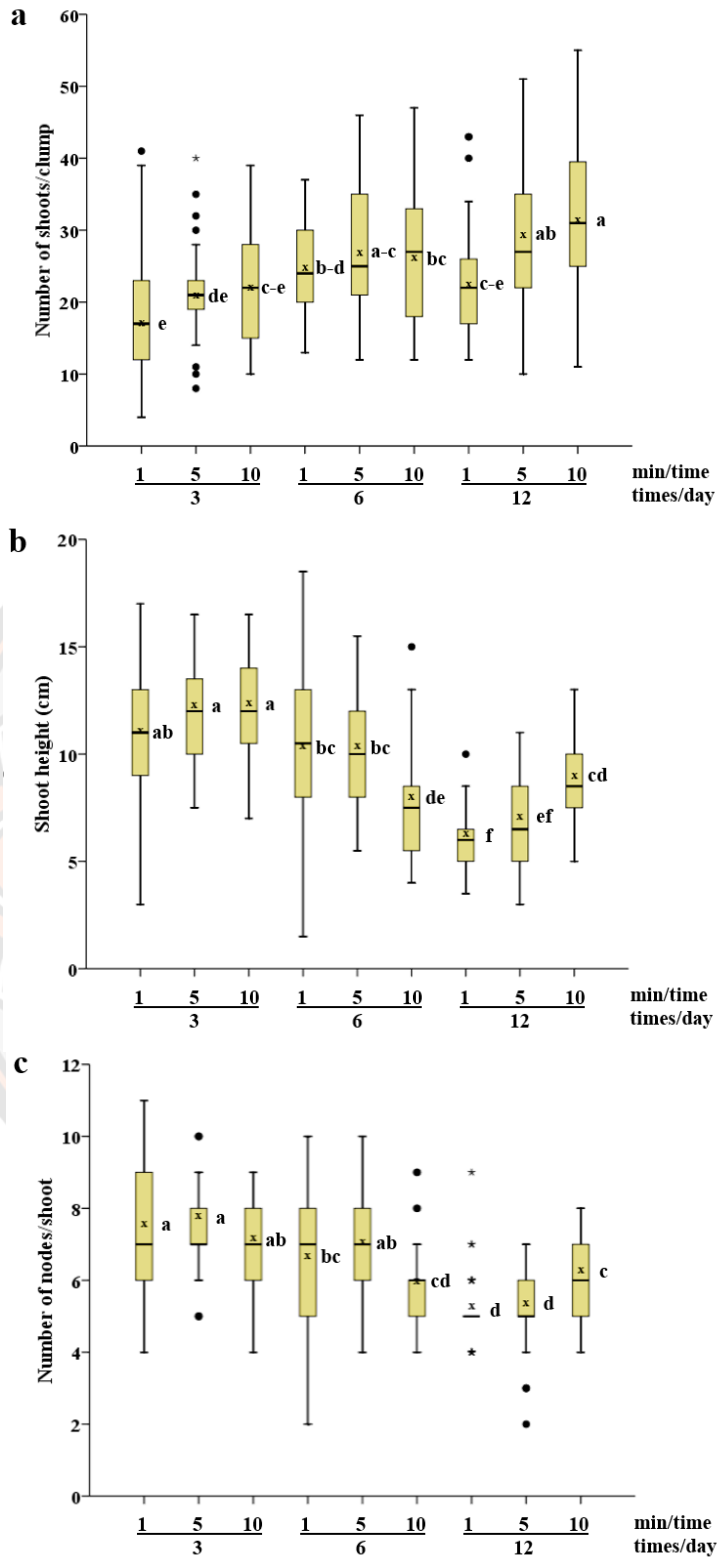

Figure 6.4 Box plots diagram represent effect of immersion frequency and duration of temporary immersion system on number of shoot per clump (a), shoot height (b) and number of node per shoot (c) of *Bacopa monnieri* after 4 weeks cultivation. The

bottom and top of the box show the 25th and 75th percentiles, respectively while the bottom and top of whiskers signify the 10th and 90th, respectively. The black dot (•) and asterisk (∗) represent the outliers and extreme values respectively. The thick horizontal line and the × symbol within the bar are the median and mean of 3 replicates (20 explants per replicate), respectively. The different letters beside the bar indicate significant differences of mean at $p \leq 0.01$ according to DMRT. Shoot height and number of nodes were recorded from the longest plantlet within the clump.

Table 6.2 Effect of immersion frequency and duration of temporary immersion system on biomass production of *Bacopa monnieri*. The data was recorded at 4 weeks after culture.

Frequency (times/day)	Duration (min/time)	Clump FW (g) [1]	Aerial part weight (g/clump)		Total DW (g/container)
			FW	DW	
3	1	2.25 ± 0.21 bc	1.46 ± 0.13 bc	0.12 ± 0.01 bcd	2.06 ± 0.77 ab
	5	2.63 ± 0.18 ab	1.76 ± 0.11 abc	0.13 ± 0.01 abc	2.33 ± 0.58 a
	10	2.74 ± 0.08 ab	1.92 ± 0.06 a	0.14 ± 0.01 abc	2.34 ± 0.24 a
6	1	2.93 ± 0.16 a	1.83 ± 0.11 ab	0.15 ± 0.01 ab	2.72 ± 0.57 a
	5	2.58 ± 0.23 abc	1.63 ± 0.17 abc	0.14 ± 0.01 abc	2.40 ± 0.79 a
	10	2.00 ± 0.09 cd	1.34 ± 0.09 c	0.11 ± 0.00 cd	1.96 ± 0.45 ab
12	1	1.52 ± 0.03 d	0.87 ± 0.16 d	0.09 ± 0.00 d	1.54 ± 0.18 b
	5	2.24 ± 0.23 bc	1.38 ± 0.18 c	0.13 ± 0.02 abc	2.24 ± 1.08 a
	10	2.94 ± 0.14 a	1.83 ± 0.11 ab	0.16 ± 0.00 a	2.42 ± 0.66 a

Values are mean ± SE of 3 replicates (20 explants per replicate) except total DW are mean ± SD of 3 containers. The same letters within the column are not significantly different at $p \leq 0.01$ according to DMRT.

[1] Clump FW was the fresh weight of whole clump including root.

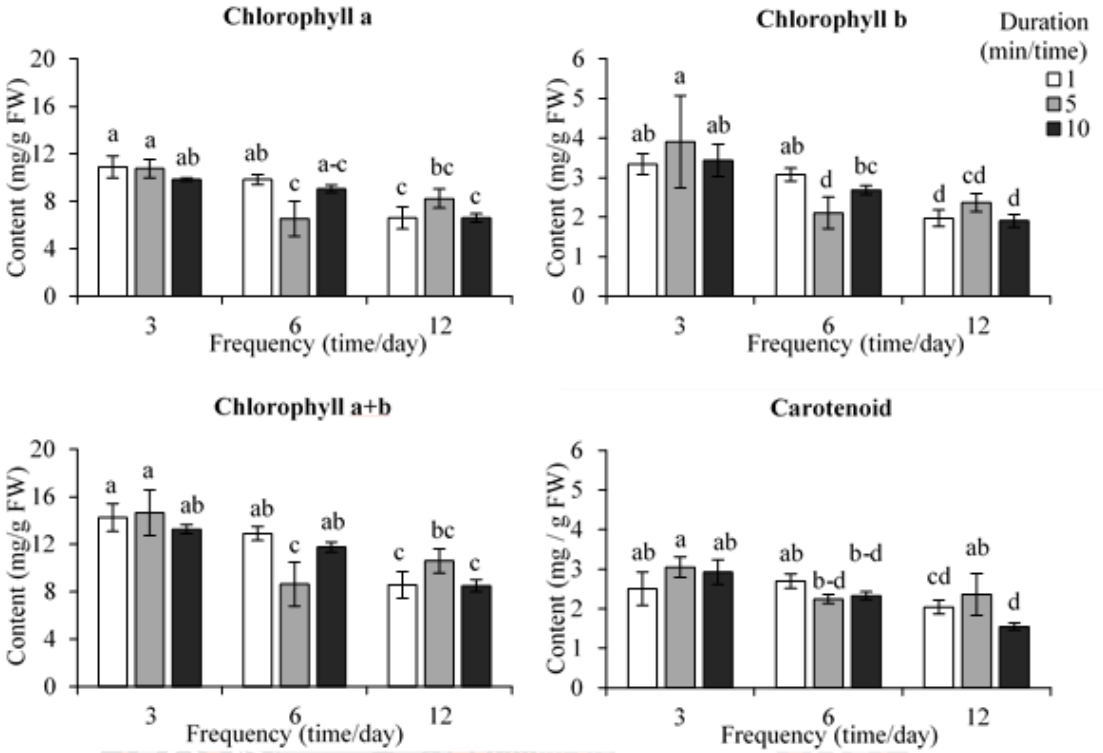

Figure 6.5 Effect of immersion frequency and duration of temporary immersion system on photosynthetic pigments of *Bacopa monnieri* after 4 weeks cultivation. Bar and error bar are mean and SE of 3 replicates (3 samples per replicate) respectively. The same letters within the same pigments are not significantly different at $p \leq 0.01$ according to DMRT.

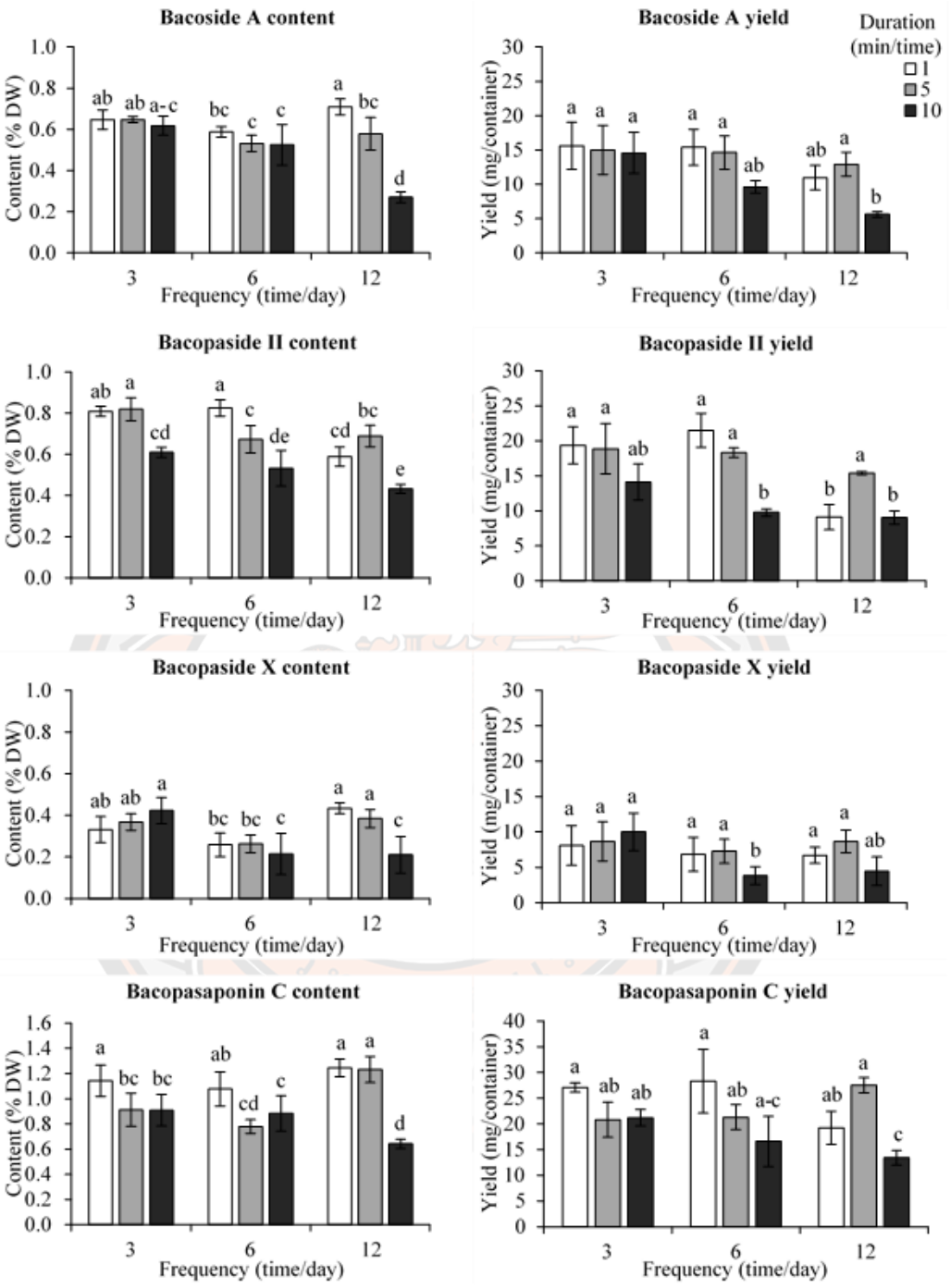

Figure 6.6 Effect of immersion frequency and duration of temporary immersion system on bacosides production of *Bacopa monnieri* after 4 weeks cultivation. Bar and error bar are mean and SD of 3 replicates (1 plant sample per replicate for content and 1 container per replicate for yield, respectively). The same letters within the same compounds are not significantly different at $p \leq 0.05$ according to DMRT.

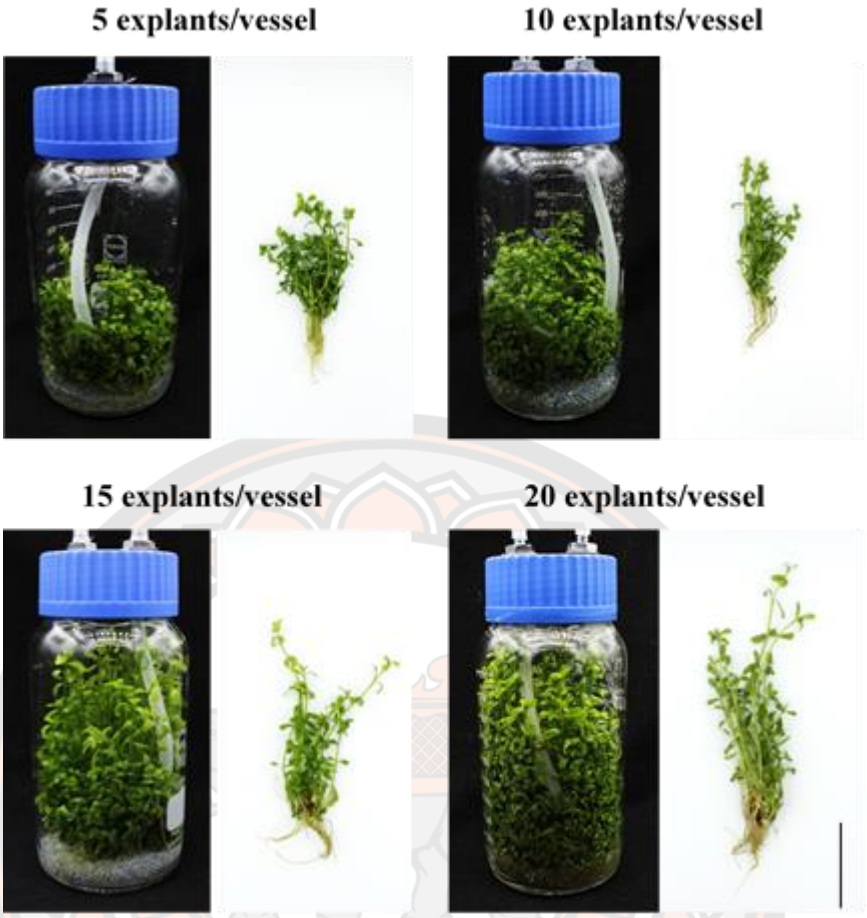

Figure 6.7 Comparative growth and development of leaf segments with regenerated shoot buds of *Bacopa monnieri* after 4 weeks cultivation in temporary immersion system (3 times/day and 10 min/time) with different inoculum densities. Bar = 5 cm.

Table 6.3 Effect of inoculum density of temporary immersion system on survival and abnormality rate of *Bacopa monnieri*. The data was recorded at 4 weeks after culture.

Inoculum (explants/vessel)	**Survival rate (%)**	**Severe leaf necrosis (%) [1]**	**Severe leaf chlorosis (%) [1]**	**Hyperhydricity (%) [2]**
5	100.0	0.0 ± 0.0 ns	73.3 ± 6.7 a	0.0 ± 0.0 ns
10	100.0	0.0 ± 0.0	53.3 ± 8.8 c	0.0 ± 0.0
15	100.0	0.0 ± 0.0	60.0 ± 13.9 b	0.0 ± 0.0
20	100.0	1.7 ± 0.0	80.0 ± 7.6 a	1.7 ± 1.7

Values are mean ± SE of 3 replicates (20 explants per replicate). The same letters within the column are not significantly different at $p \leq 0.01$ according to DMRT, ns; non-significant difference.

[1] Severe leaf necrosis and leaf chlorosis were scored when plantlet within clump show severe symptoms (**Figure 6.3 b** and **c**).

[2] Hyperhydricity was observed from leaves at the base of clump.

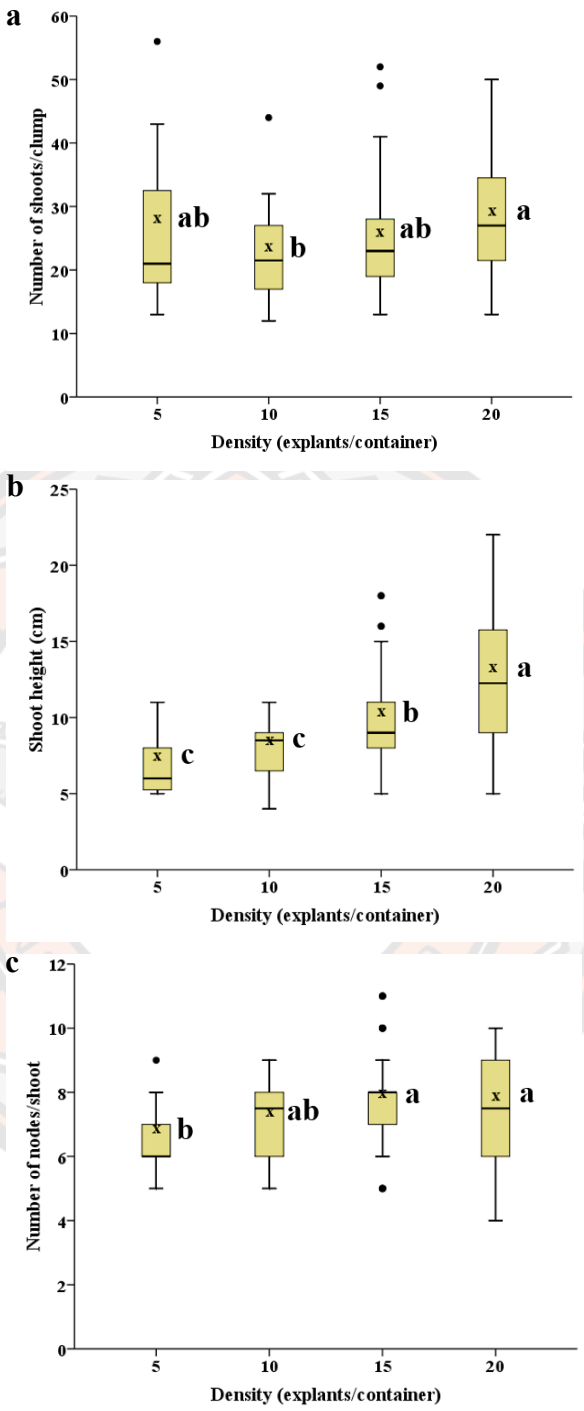

Figure 6.8 Box plots diagram represent effect of inoculum density of temporary immersion system on number of shoot per clump (a), shoot height (b) and number of node per shoot (c) of Bacopa monnieri after 4 weeks cultivation. The bottom and top of the box show the 25th and 75th percentiles, respectively while the bottom and top of whiskers signify the 10th and 90th, respectively. The black dot (•) represent the

outliers. The thick horizontal line and the × symbol within the bar are the median and mean of 3 replicates (20 explants per replicate), respectively. The different letters beside the bar indicate significantly different of mean at $p \leq 0.01$ according to DMRT. Shoot height and number of nodes were recorded from the longest plantlet within the clump.

Table 6.4 Effect of inoculum density of temporary immersion system on biomass production of *Bacopa monnieri* after 4 weeks cultivation.

Inoculum (explants/vessel)	Clump FW (g) [1]	Aerial part weight (g/clump)		Total DW (g/container)
		FW	DW	
5	2.80 ± 0.74 a	1.82 ± 0.56 ab	0.14 ± 0.03 ns	0.68 ± 0.36 c
10	2.03 ± 0.01 b	1.39 ± 0.01 b	0.11 ± 0.00	1.08 ± 0.06 c
15	2.36 ± 0.21 ab	1.58 ± 0.19 b	0.13 ± 0.01	1.96 ± 0.46 b
20	2.92 ± 0.27 a	2.14 ± 0.21 a	0.14 ± 0.01	3.01 ± 0.53 a

Values are mean ± SE of 3 replicates (20 explants per replicate) except total DW are mean ± SD of 3 containers. The same letters within the column are not significantly different at $p \leq 0.01$ according to DMRT, ns; non-significant difference.

[1] Clump FW was the fresh weight of whole clump including root.

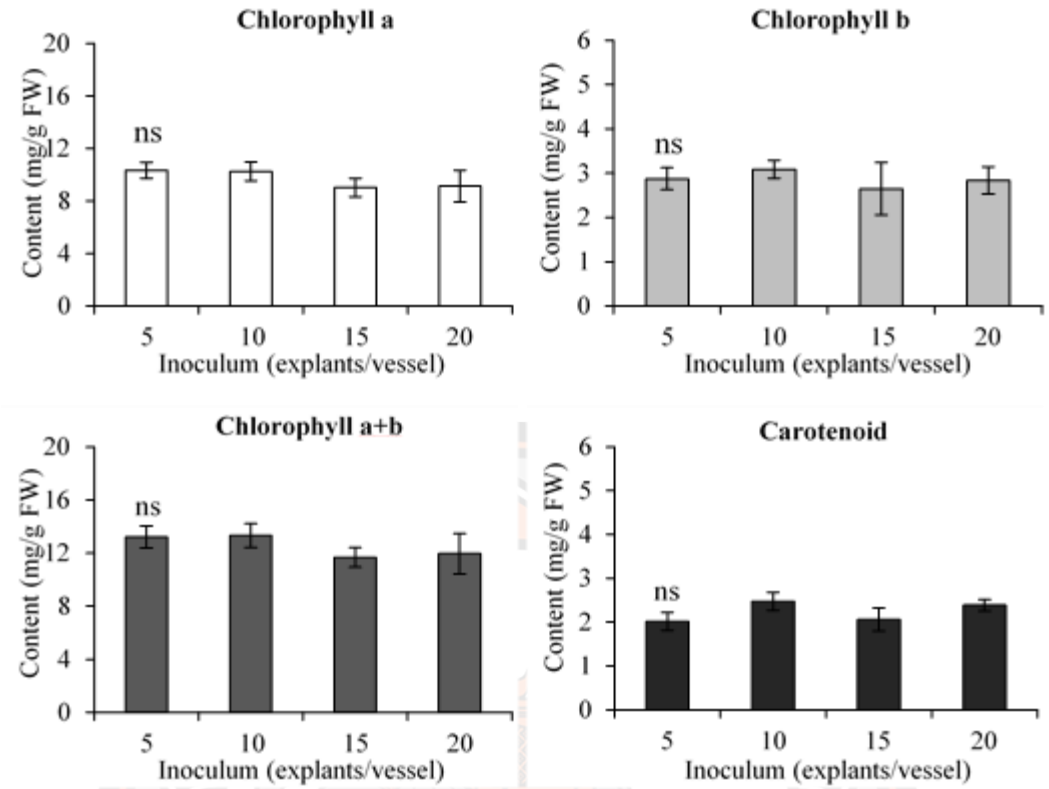

Figure 6.9 Effect of inoculum density of temporary immersion system on photosynthetic pigments of *Bacopa monnieri* after 4 weeks cultivation. Bar and error bar are mean and SD of 3 replicates (3 samples per replicate) respectively, ns indicate not significantly different at $p \leq 0.05$ according to DMRT.

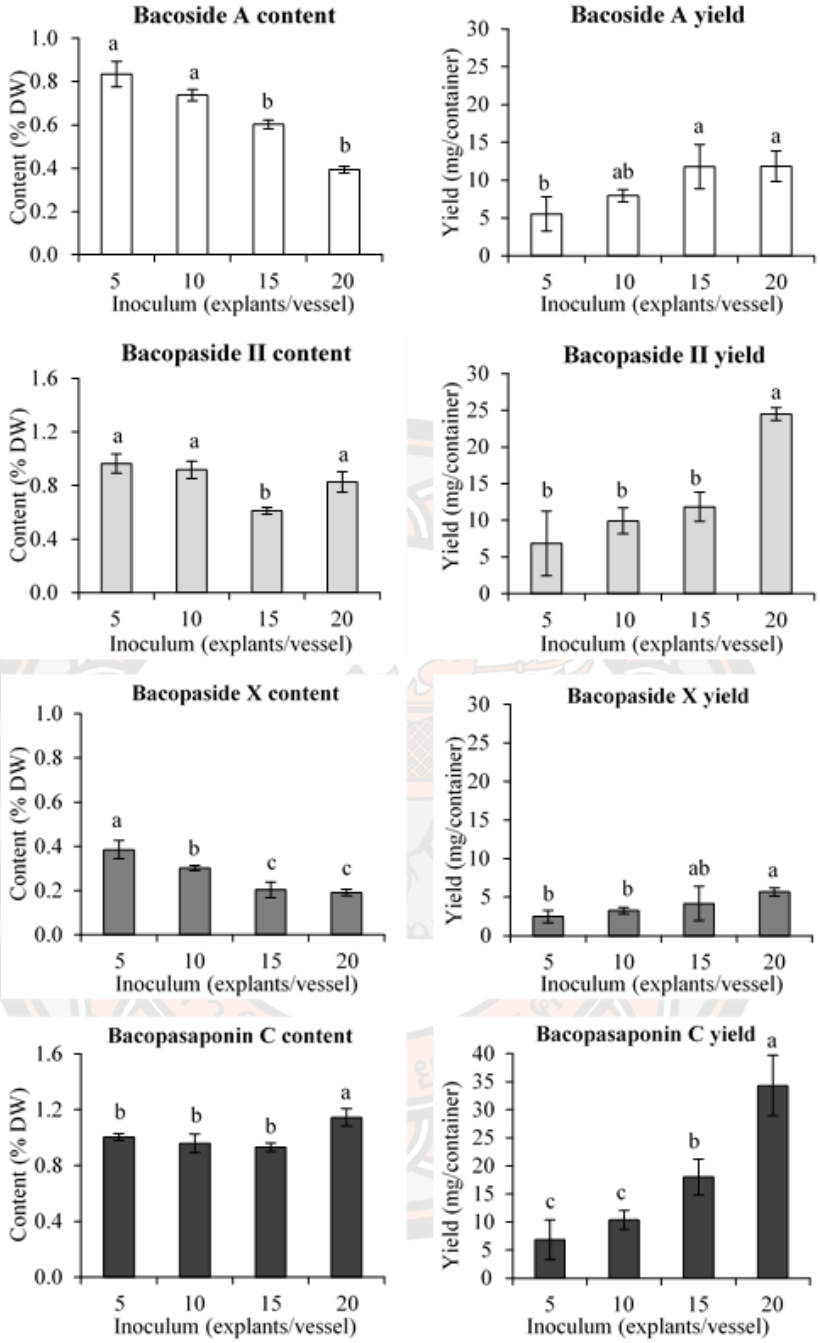

Figure 6.10 Effect of inoculum density of temporary immersion system (3 time/day and 10 min/time) on bacoside production of *Bacopa monnieri* after 4 weeks cultivation. Bar and error bar are mean and SD of 3 replicates (1 sample per replicate for content and 1 container per replicate for yield), respectively. The same letters within the same compounds are not significantly different at $p \leq 0.05$ according to DMRT.

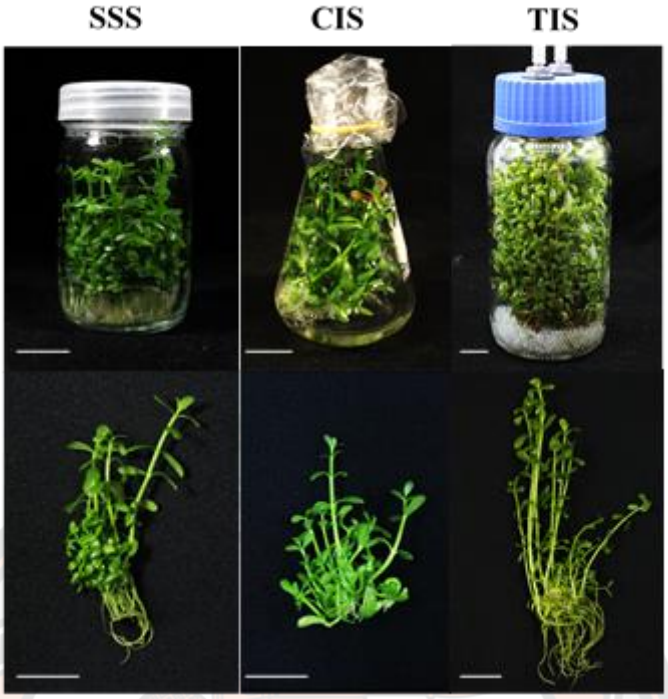

Figure 6.11 Comparative growth and development of leaf segments with regenerated shoot buds of *Bacopa monnieri* after 4 weeks cultured in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) systems. TIS was selected from the best results of previous experiment (20 explants/vessel, medium feeding 3 times/day and 10 min/time). Bar = 2 cm.

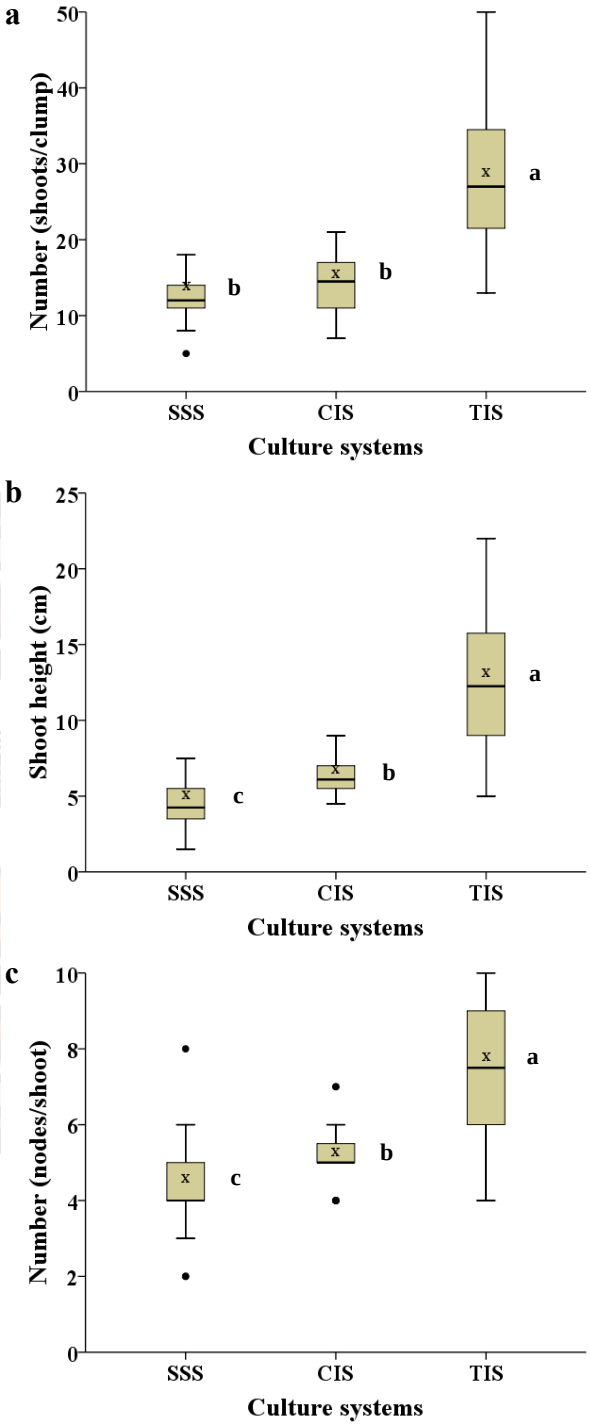

Figure 6.12 Box plots diagram represent number of shoots per clump (a), shoot height (b) and number of nodes per clump (c) of *Bacopa monnieri* after 4 weeks cultured in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) system. TIS was selected from the best results of previous experiment (20

explants/vessel, medium feeding 3 times/day and 10 min/time). The bottom and top of the box show the 25th and 75th percentiles, respectively while the bottom and top of whiskers signify the 10th and 90th, respectively. The black dot (•) represent the outliers. The thick horizontal line and the × symbol within the bar are the median and mean of 3 replicates (20 explants per replicate), respectively. The different letters beside the bar indicate significant difference at $p \leq 0.01$ according to DMRT. Shoot height and number of nodes were recorded from the longest plantlet within the clump.

Table 6.5 Effect of culture systems biomass production of *Bacopa monnieri* after 4 weeks cultivation

Culture systems [1]	**Clump FW (g)** [2]	**Aerial part weight (g/clump)**		**Total DW (g/container)**
		FW	**DW**	
SSS	1.16 ± 0.05 b	1.02 ± 0.04 b	0.10 ± 0.00 b	0.19 ± 0.03 b
CIS	1.34 ± 0.03 b	1.21 ± 0.02 b	0.10 ± 0.00 b	0.19 ± 0.01 b
TIS	2.92 ± 0.27 a	2.14 ± 0.21 a	0.14 ± 0.01 a	3.01 ± 0.53 a

Values are mean ± SE of 3 replicates (20 explants per replicate) except total DW are mean ± SD of 3 containers. The same letters within the column are not significantly different at $p \leq 0.01$ according to DMRT. For semi-solid and continuous immersion systems, the first 3 containers showing highest dry weight were selected.

[1] SSS, semi-solid system; CIS, continuous immersion system; TIS, temporary immersion system (20 explants/vessel, medium feeding 3 times/day and 10 min/time).

[2] Clump FW was the fresh weight of whole clump including root.

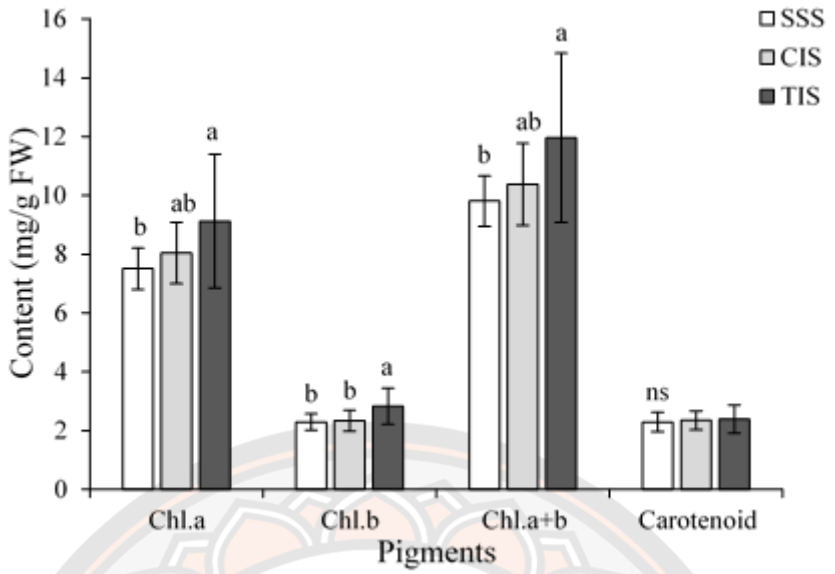

Figure 6.13 Chlorophyll a (Chl.a), Chlorophyll b (Chl.b), Chlorophyll a+b (Chl.a+b) and Carotenoid of *Bacopa monnieri* after 4 weeks cultured in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) system (20 explants/vessel, medium feeding 3 times/day and 10 min/time). Bar and error bar are mean and SD of 3 replicates (3 sample per replicate), respectively. The same letters within the same pigments are not significantly different at $p \leq 0.05$ according to DMRT, ns; non-significant difference.

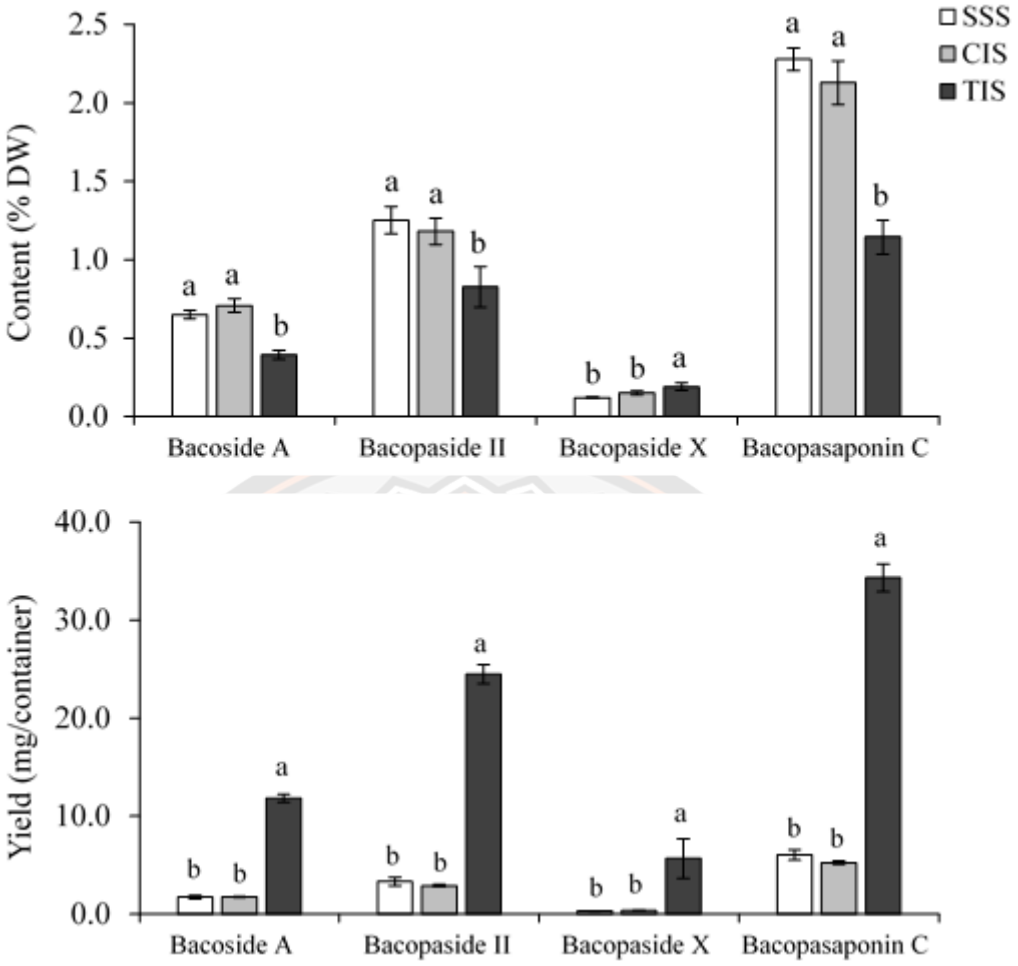

Figure 6.14 Bacosides production of *Bacopa monnieri* after 4 weeks cultured in semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS) system (20 explants/vessel, medium feeding 3 times/day and 10 min/time). Bar and error bar are mean and SD of 3 replicates (1 sample per replicate for content and 1 container per replicate for yield), respectively. The same letters within the same compounds are not significantly different at $p \leq 0.01$ according to DMRT.

CHAPTER VII

Discussion

Plant tissue culture technologies have been recognized as a powerful tool for propagation of many plant species. Various approaches of this technology are not only using conventional techniques with small culture container but also using innovative cultivation methods for large scale production. Large scale production using temporary immersion system (TIS) has been developed and improved to become the most effective system in many plant cultivation, such as commercial crops (Roels et al., 2006; Scherer et al., 2015; B. Zhang et al., 2018), medicinal herbs (Georgiev et al., 2014; Langhansova, Marsik, & Vanek, 2012; Mišić et al., 2013), and endangered plant species (Masnoddin, Repin, & Aziz, 2016; Wu, Baque, & Paek, 2010). The core concept of TIS is to avoid consistently continuous immersion of the explants. Several studies have been reported the achievement of developing TIS and indicated that TIS is superior to other culture systems especially for mass propagation (An et al., 2016; Businge, Trifonova, Schneider, Rödel, & Egertsdotter, 2017; Sharma et al., 2015). Moreover, simplified and practicable TIS was continuously developed by modification of alternative materials (An et al., 2016; Moreira et al., 2013; Topoonyanont et al., 2017). In this research, the completely twin-bottles TIS was successfully established in the final development version for further comfortable and flexible utilization whereas the efficient function of versatile TIS had been accurately investigated by comparative culture systems for mass propagation of some valuable plant species including *Epipactis flava*, *Calanthe rubens*, *Bacopa monnieri* and *Drosera communis*. TIS technology is helpful to improve not only quantity but also quality of plant production. The positive effect of TIS was revealed in term of growth proliferation, biomass accumulation and yield. However, this TIS condition might be not suitable for mass production of *C. rubens* using pseudo-bulblets. In this case, the microenvironment influence on plant growth and increasing carbon metabolism (Georgiev et al., 2014) as well as the main factors affecting in TIS still needs to be concerned and studied. In addition, the direct exposure of explant in our invented TIS

approach to simplify the oxygen transport from the gas to plant cells (Roels et al., 2006) must also mentioned and improved. Furthermore, the photomixotrophic process of in vitro culture might be occurred during cultivation in TIS (Maritza Escalona et al., 2003; Etienne & Berthouly, 2002), which leaded to enhance growth and development of plant better than cultured by the conventional cultivation systems.

CHAPTER VIII

Summary

This research presented that an innovative system using twin-bottles temporary immersion system could provide mass production efficiency of some valuable plant species in term of growth performance, biomass accumulation and yield higher than conventional cultivation systems both semi-solid and continuous immersion system. Temporary immersion system developed from this research was not only the most efficient and suitable method for mass propagation of *E. flava* and providing the highest biomass, shoot multiplication and plumbagin yield of *D. communis* but also influencing on growth, morphology and bacoside production of *B. monnieri* cultured with different immersion conditions. However, some of immersion conditions may not be suitable for growth and development of *C. rubens* using pseudo-bulblets explants. In addition, the development of simplified temporary immersion system in this research provided flexible operation, easy to handle and convenient modification for further exploration.

REFERENCES

Abràmoff, M. D., Magalhães, P. J. & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics international, 11*(7), 36-42.

Affreen, F. (2008). *Temporary immersion bioreactor* (S. D. G. a. Y. Ibaraki Ed.): Springer.

Afreen, F. (2006). Temporary Immersion Bioreactor. In S. D. Gupta & Y. Ibaraki (Eds.), *Plan Tissue Culture Engineering* (pp. 187-201). Dordrecht: Springer Netherlands.

Ahmadian, M., Babaei, A., Shokri, S. & Hessami, S. (2017). Micropropagation of carnation (*Dianthus caryophyllus* L.) in liquid medium by temporary immersion bioreactor in comparison with solid culture. *Journal of Genetic Engineering and Biotechnology, 15*(2), 309-315. doi:10.1016/j.jgeb.2017.07.005

Aitken-Christie, J. (1991). Automation *Micropropagation* (pp. 363-388): Springer.

Akdemir, H., Süzerer, V., Onay, A., Tilkat, E., Ersali, Y. & Çiftçi, Y. O. (2014). Micropropagation of the pistachio and its rootstocks by temporary immersion system. *Plant Cell, Tissue and Organ Culture, 117*(1), 65-76. doi:10.1007/s11240-013-0421-0

Akin-Idowu, P. E., Ibitoye, D. O. & Ademoyegun, O. T. (2009). Tissue culture as a plant production technique for horticultural crops. *African Journal of Biotechnology, 8*, 3782-3788.

Al-Snafi, A. E. (2013). The pharmacology of Bacopa monniera. A review. *International Journal of Pharma Sciences and Research, 4*(12), 154-159.

Alvard, D., Cote, F. & Teisson, C. (1993). Comparison of methods of liquid medium culture for banana micropropagation. *Plant Cell, Tissue and Organ Culture, 32*(1), 55-60.

An, C. H., Kim, Y. W., Moon, H. K. & Yi, J. S. (2016). Effects of in vitro culture types on regeneration and acclimatization of yellow poplar (*Liriodendron tulipifera* L.) from somatic embryos. *Journal of Plant Biotechnology, 43*(1), 110-118. doi:10.5010/jpb.2016.43.1.110

Aragón, C., Sánchez, C., Gonzalez-Olmedo, J., Escalona, M., Carvalho, L. & Amâncio, S. (2014). Comparison of plantain plantlets propagated in temporary immersion

bioreactors and gelled medium during in vitro growth and acclimatization. *Biologia Plantarum, 58*(1), 29-38.

Aragón, C. E., Escalona, M., Capote, I., Pina, D., Cejas, I., Rodriguez, R., . . . Debergh, P. (2005). Photosynthesis and carbon metabolism in plantain (*Musa* AAB) plantlets growing in temporary immersion bioreactors and during ex vitro acclimatization. *In Vitro Cellular and Developmental Biology-Plant, 41*(4), 550-554.

Aragón, C. E., Escalona, M., Rodriguez, R., Cañal, M. J., Capote, I., Pina, D. & González-Olmedo, J. (2010). Effect of sucrose, light, and carbon dioxide on plantain micropropagation in temporary immersion bioreactors. *In Vitro Cellular and Developmental Biology-Plant, 46*(1), 89-94.

Arencibia, A. D., Vergara, C., Quiroz, K., Carrasco, B., Bravo, C. & Garcia-Gonzales, R. (2013). An Approach for Micropropagation of Blueberry (*Vaccinium corymbosum* L.) Plants Mediated by Temporary Immersion Bioreactors (TIBs). *American Journal of Plant Sciences, 04*(05), 1022-1028. doi:10.4236/ajps.2013.45126

Arencibia, A. D., Vergara, C., Quiroz, K., Carrasco, B. & García-Gonzales, R. (2013). Establishment of photomixotrophic cultures for raspberry micropropagation in Temporary Immersion Bioreactors (TIBs). *Scientia Horticulturae, 160*, 49-53. doi:10.1016/j.scienta.2013.05.010

Averyanov, L. V. (2007). New species of orchids from Vietnam. *Taiwania, 52*(4), 287-306.

Bansal, M., Reddy, M. S. & Kumar, A. (2016). Seasonal variations in harvest index and bacoside A contents amongst accessions of *Bacopa monnieri* (L.) Wettst. collected from wild populations. *Physiology and Molecular Biology of Plants, 22*(3), 407-413. doi:10.1007/s12298-016-0366-y

Baranyai, B., Bäcker, C., Reich, C. & Lindequist, U. (2016). The production of 7-methyljuglone, plumbagin and quercetin in wild and cultivated *Drosera rotundifolia* and *Drosera intermedia*. *Mires and Peat, 18*(19), 1-8.

Barthlott, W. & Ashdown, M. (2007). *curious world of carnivorous plants*: Timber Press.

Bello-Bello, J. J., Canto-Flick, A., Balam-Uc, E., Gómez-Uc, E., Robert, M. L., Iglesias-Andreu, L. G. & Santana-Buzzy, N. (2010). Improvement of in vitro proliferation and elongation of *Habanero* pepper shoots (*Capsicum chinense* Jacq.) by temporary immersion. *HortScience, 45*(7), 1093-1098.

Bellusci, F., Pellegrino, G. & Musacchio, A. (2009). Different levels of inbreeding depression between outcrossing and selfing *Serapias* species. *Biologia Plantarum, 53*(1), 175-178.

Berthouly, M. & Etienne, H. (2005). Temporary immersion system: a new concept for use liquid medium in mass propagation *Liquid culture systems for in vitro plant propagation* (pp. 165-195): Springer.

Bhojwani, S. & Razdan, M. (1996). Plant tissue culture, theory and practices. A revised edition. *Studies in plant science, 5*.

Boufford, D. E. & Zhang, W. (2007). *Spiranthes sunii* Boufford & Wenheng Zhang, sp. nov.; a new rheophytic orchid from Gansu Province, China. *Harvard papers in botany, 13*(2), 261-267.

Businge, E., Trifonova, A., Schneider, C., Rödel, P. & Egertsdotter, U. (2017). Evaluation of a New Temporary Immersion Bioreactor System for Micropropagation of Cultivars of *Eucalyptus*, Birch and Fir. *Forests, 8*(6), 196. doi:10.3390/f8060196

Chakrabarty, D., Dewir, Y. H., Hahn, E. J., Datta, S. K. & Paek, K. Y. (2006). The dynamics of nutrient utilization and growth of apple root stock ‘M9 EMLA’ in temporary versus continuous immersion bioreactors. *Plant Growth Regulation, 51*(1), 11-19. doi:10.1007/s10725-006-9115-5

Cribb, P. J., Kell, S. P., Dixon, K. W. & Barrett, R. L. (2003). Orchid conservation: a global perspective. *Orchid conservation. Natural History Publications, Kota Kinabalu*, 1-24.

De, U., Son, J. Y., Jeon, Y., Ha, S.-Y., Park, Y. J., Yoon, S., . . . Kim, I. S. (2019). Plumbagin from a tropical pitcher plant (*Nepenthes alata* Blanco) induces apoptotic cell death via a p53-dependent pathway in MCF-7 human breast cancer cells. *Food and Chemical Toxicology, 123*, 492-500.

Debnath, S. (2011). Bioreactors and molecular analysis in berry crop micropropagation

– A review. *Canadian Journal of Plant Science, 91*(1), 147-157. doi:10.4141/cjps10131

Decruse, S. W., Reny, N., Shylajakumari, S. & Krishnan, P. (2013). In vitro propagation and field establishment of *Eulophia cullenii* (Wight) Bl., a critically endangered orchid of Western Ghats, India through culture of seeds and axenic seedling-derived rhizomes. *In Vitro Cellular and Developmental Biology-Plant, 49*(5), 520-528.

Dewir, Y. H., Chakrabarty, D., Ali, M. B., Hahn, E. J. & Paek, K. Y. (2006). Lipid peroxidation and antioxidant enzyme activities of *Euphorbia millii* hyperhydric shoots. *Environmental and Experimental Botany, 58*(1-3), 93-99. doi:10.1016/j.envexpbot.2005.06.019

Diengdoh, R. V., Kumaria, S., Tandon, P. & Das, M. C. (2017). Asymbiotic germination and seed storage of *Paphiopedilum insigne*, an endangered lady's slipper orchid. *South African Journal of Botany, 112*, 215-224.

Dohling, S., Kumaria, S. & Tandon, P. (2012). Multiple shoot induction from axillary bud cultures of the medicinal orchid, *Dendrobium longicornu*. *AoB Plants, 2012*.

Ducos, J. P., Labbe, G., Lambot, C. & Pétiard, V. (2007). Pilot scale process for the production of pre-germinated somatic embryos of selected robusta (*Coffea canephora*) clones. *In Vitro Cellular and Developmental Biology - Plant, 43*(6), 652-659. doi:10.1007/s11627-007-9075-0

Ducos, J. P., Terrier, B., Courtois, D. & Pétiard, V. (2008). Improvement of plastic-based disposable bioreactors for plant science needs. *Phytochemistry Reviews, 7*(3), 607-613. doi:10.1007/s11101-008-9089-1

Dutra, D., Johnson, T. R., Kauth, P. J., Stewart, S. L., Kane, M. E. & Richardson, L. (2008). Asymbiotic seed germination, in vitro seedling development, and greenhouse acclimatization of the threatened terrestrial orchid *Bletia purpurea*. *Plant Cell, Tissue and Organ Culture, 94*(1), 11-21.

Dutra, D., Kane, M. E., Adams, C. R. & Richardson, L. (2009). Reproductive biology of *Cyrtopodium punctatum* in situ: implications for conservation of an endangered Florida orchid. *Plant species biology, 24*(2), 92-103.

Escalona, M., Lorenzo, J., González, B., Daquinta, M., González, J., Desjardins, Y. &

Borroto, C. (1999). Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. *Plant Cell Reports, 18*(9), 743-748.

Escalona, M., Samson, G., Borroto, C. & Desjardins, Y. (2003). Physiology of effects of temporary immersion bioreactors on micropropagated pineapple plantlets. *In Vitro Cellular and Developmental Biology - Plant, 39*(6), 651-656. doi:10.1079/ivp2003473

Esyanti, R. R., Adhitama, N. & Manurung, R. (2016). Efficiency Evaluation of Vanda Tricolor Growth in Temporary Immerse System Bioreactor and Thin Layer Culture System. *Journal of Advanced Agricultural Technologies, 3*(1), 63-66. doi:10.18178/joaat.3.1.63-66

Etienne, H. & Berthouly, M. (2002). Temporary immersion systems in plant micropropagation. *Plant Cell, Tissue and Organ Culture, 69*(3), 215-231.

Ferreira, D. T., Andrei, C. C., Saridakis, H. O., Faria, T. d. J., Vinhato, E., Carvalho, K. E., . . . Braz-Filho, R. (2004). Antimicrobial activity and chemical investigation of Brazilian *Drosera*. *Memorias do Instituto Oswaldo Cruz, 99*(7), 753-755.

Fukao, T. & Bailey-Serres, J. (2004). Plant responses to hypoxia–is survival a balancing act? *Trends in plant science, 9*(9), 449-456.

Gao, M., Jiang, W., Wei, S., Lin, Z., Cai, B., Yang, L., . . . Chen, L. (2015). High-efficiency propagation of Chinese water chestnut [*Eleocharis dulcis* (Burm.f.) Trin. ex Hensch] using a temporary immersion bioreactor system. *Plant Cell, Tissue and Organ Culture, 121*(3), 761-772. doi:10.1007/s11240-015-0732-4

George, E. F., Hall, M. A. & De Klerk, G.-J. (2008). The components of plant tissue culture media I: macro-and micro-nutrients *Plant propagation by tissue culture* (pp. 65-113): Springer.

Georgiev, V., Ilieva, M., Bley, T. & Pavlov, A. (2008). Betalain production in plant in vitro systems. *Acta Physiologiae Plantarum, 30*(5), 581-593. doi:10.1007/s11738-008-0170-6

Georgiev, V., Ivanov, I., Berkov, S. & Pavlov, A. (2013). Temporary immersion systems for Amaryllidaceae alkaloids biosynthesis by *Pancratium maritimum* L. shoot culture. *Journal of Plant Biochemistry and Biotechnology, 23*(4), 389-398. doi:10.1007/s13562-013-0222-x

Georgiev, V., Schumann, A., Pavlov, A. & Bley, T. (2014). Temporary immersion systems in plant biotechnology. *Engineering in Life Sciences, 14*(6), 607-621. doi:10.1002/elsc.201300166

Godo, T., Komori, M., Nakaoki, E., Yukawa, T. & Miyoshi, K. (2010). Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture in vitro. *In Vitro Cellular and Developmental Biology-Plant, 46*(3), 323-328.

Godoy, S., Tapia, E., Seit, P., Andrade, D., Sánchez, E., Andrade, P., . . . Prieto, H. (2017). Temporary immersion systems for the mass propagation of sweet cherry cultivars and cherry rootstocks: development of a micropropagation procedure and effect of culture conditions on plant quality. *In Vitro Cellular and Developmental Biology - Plant, 53*(5), 494-504. doi:10.1007/s11627-017-9856-z

Graham, I. A., Besser, K., Blumer, S., Branigan, C. A., Czechowski, T., Elias, L., . . . Khan, A. M. (2010). The genetic map of *Artemisia annua* L. identifies loci affecting yield of the antimalarial drug artemisinin. *Science, 327*(5963), 328-331.

Grzegorczyk-Karolak, I., Rytczak, P., Bielecki, S. & Wysokińska, H. (2017). The influence of liquid systems for shoot multiplication, secondary metabolite production and plant regeneration of *Scutellaria alpina*. *Plant Cell, Tissue and Organ Culture, 128*(2), 479-486.

Gutiérrez, L. G., López-Franco, R. & Morales-Pinzón, T. (2016). Micropropagation of *Guadua angustifolia* Kunth (Poaceae) using a temporary immersion system RITA®. *African Journal of Biotechnology, 15*(28), 1503-1510.

Hahn, E.-J. & Paek, K.-Y. (2005). Multiplication of *Chrysanthemum* shoots in bioreactors as affected by culture method and inoculation density of single node stems. *Plant Cell, Tissue and Organ Culture, 81*(3), 301-306. doi:10.1007/s11240-004-6655-0

Hajong, S., Kumaria, S. & Tandon, P. (2010). In vitro propagation of the medicinal orchid *Dendrobium chrysanthum*. *Proceedings of the Indian National Science Academy-Part A: Physical Sciences, 76*(2), 67.

Hanhineva, K. J. & Karenlampi, S. O. (2007). Production of transgenic strawberries by

temporary immersion bioreactor system and verification by TAIL-PCR. *BMC Biotechnol, 7*, 11. doi:10.1186/1472-6750-7-11

Haque, S. M., Chakraborty, A., Dey, D., Mukherjee, S., Nayak, S. & Ghosh, B. (2017). Improved micropropagation of *Bacopa monnieri* (L.) Wettst. (Plantaginaceae) and antimicrobial activity of in vitro and ex vitro raised plants against multidrug-resistant clinical isolates of urinary tract infecting (UTI) and respiratory tract infecting (RTI) bacteria. *Clinical Phytoscience*, *3*(1). doi:10.1186/s40816-017-0055-6

Harris, R. E. & Masson, E. B. (1983). Two machines for in vitro propagation of plants in liquid media. *Canadian Journal of Plant Science, 63*(1), 311-316.

Harvais, G. (1982). An improved culture medium for growing the orchid *Cypripedium reginae* axenically. *Canadian Journal of botany, 60*(12), 2547-2555.

Hazarika, B. (2003). Acclimatization of tissue-cultured plants. *Current Science*, 1704-1712.

Hebert, L. E., Weuve, J., Scherr, P. A. & Evans, D. A. (2013). Alzheimer disease in the United States (2010–2050) estimated using the 2010 census. *Neurology, 80*(19), 1778-1783.

Hsu, R. C.-C. & Lee, Y.-I. (2012). Seed development of *Cypripedium debile* Rchb. f. in relation to asymbiotic germination. *HortScience, 47*(10), 1495-1498.

Ibrahim, M. H., Chee Kong, Y. & Mohd Zain, N. A. (2017). Effect of Cadmium and Copper Exposure on Growth, Secondary Metabolites and Antioxidant Activity in the Medicinal Plant Sambung Nyawa (*Gynura procumbens* (Lour.) Merr). *Molecules*, *22*(10). doi:10.3390/molecules22101623

Ibrahim, R. (2017). The potential of bioreactor technology for large-scale plant micropropagation. *Acta Horticulturae*, (1155), 573-584. doi:10.17660/ActaHortic.2017.1155.84

Isah, T. (2015). Adjustments to In Vitro Culture Conditions and Associated Anomalies in Plants. *Acta Biologica Cracoviensia Botanica, 57*(2), 9-28. doi:10.1515/abcsb-2015-0026

Isah, T., Umar, S., Mujib, A., Sharma, M. P., Rajasekharan, P. E., Zafar, N. & Frukh, A. (2017). Secondary metabolism of pharmaceuticals in the plant in vitro cultures:

strategies, approaches, and limitations to achieving higher yield. *Plant Cell, Tissue and Organ Culture, 132*(2), 239-265. doi:10.1007/s11240-017-1332-2

Ivanov, I., Georgiev, V., Georgiev, M., Ilieva, M. & Pavlov, A. (2011). Galanthamine and related alkaloids production by *Leucojum aestivum* L. shoot culture using a temporary immersion technology. *Applied Biochemistry and Biotechnology, 163*(2), 268-277. doi:10.1007/s12010-010-9036-7

Jayaram, K. & Prasad, M. N. V. (2007). Rapid in vitro multiplication of *Drosera indica* L.: a vulnerable, medicinally important insectivorous plant. *Plant Biotechnology Reports, 1*(2), 79-84. doi:10.1007/s11816-007-0014-7

Jesionek, A., Kokotkiewicz, A., Wlodarska, P., Zabiegala, B., Bucinski, A. & Luczkiewicz, M. (2017). Bioreactor shoot cultures of *Rhododendron tomentosum* (Ledum palustre) for a large-scale production of bioactive volatile compounds. *Plant Cell, Tissue and Organ Culture, 131*(1), 51-64. doi:10.1007/s11240-017-1261-0

Johnson, T. R., Stewart, S. L., Dutra, D., Kane, M. E. & Richardson, L. (2007). Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)—preliminary evidence for the symbiotic culture advantage. *Plant Cell, Tissue and Organ Culture, 90*(3), 313.

Jova, M. C., Kosky, R. G. & Cuellar, E. E. (2011). Effect of liquid media culture systems on yam plant growth (*Dioscorea alata* L.'Pacala Duclos'). *Biotechnologie, Agronomie, Société et Environnement, 15*(4), 515.

Kaur, J., Nautiyal, K. & Pant, M. (2013). In vitro propagation of *Bacopa monnieri* (L.) Wettst A medicinally priced herb. *International Journal of Current Microbiology and Applied Sciences 2*(8), 131-138.

Kauth, P. (2005). *In vitro seed germination and seedling development of Calopogon tuberosus and Sacoila lanceolata var. lanceolata: Two Florida native terrestrial orchids.* University of Florida.

Kauth, P. J., Vendrame, W. A. & Kane, M. E. (2006). In vitro seed culture and seedling development of *Calopogon tuberosus*. *Plant Cell, Tissue and Organ Culture, 85*(1), 91-102.

Knudson, L. (1946). A new nutrient solution for the germination of orchid seed.

American Orchid Society Bulletin, 15, 214-217.

Kokotkiewicz, A., Bucinski, A. & Luczkiewicz, M. (2015). Xanthone, benzophenone and bioflavonoid accumulation in *Cyclopia genistoides* (L.) Vent. (honeybush) shoot cultures grown on membrane rafts and in a temporary immersion system. *Plant Cell, Tissue and Organ Culture*, *120*(1), 373-378.

Kongbangkerd, A., Homchan, S. & Promthep, K. (2011). Effects of plant growth regulators and plant growth retardants on growth and development on in vitro leaf culture of *Drosera communis* St.Hil.. *Naresuan University Science Journal, 8*(1), 87-100.

Kumar, A., Nandi, S., Bag, N. & Palni, L. (2002). Tissue culture studies in two important orchid taxa: *Rhynchostylis retusa* (L.) Bl. and *Cymbidium elegans* Lindl. *Role of plant tissue culture in biodiversity conservation and economic development. Nainital,* 113-124.

Kurzweil, H. (2010). A precursory study of the *Calanthe* group (Orchidaceae) in Thailand. *Adansonia, 32*(1), 57-108.

Langhansova, L., Marsik, P. & Vanek, T. (2012). Regulation of tissue differentiation by plant growth regulators on tTCLs of *Panax ginseng* adventitious roots. *Industrial Crops and Products, 35*(1), 154-159.

Lee, Y.-I., Lu, C.-F., Chung, M.-C., Yeung, E. C. & Lee, N. (2007). Developmental changes in endogenous abscisic acid concentrations and asymbiotic seed germination of a terrestrial orchid, *Calanthe tricarinata* Lindl. *Journal of the American Society for Horticultural Science, 132*(2), 246-252.

Leporatti, M. L. & Ivancheva, S. (2003). Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy. *Journal of Ethnopharmacology, 87*(2-3), 123-142.

Likhitwitayawuid, K., Kaewamatawong, R., Ruangrungsi, N. & Krungkrai, J. (1998). Antimalarial naphthoquinones from *Nepenthes thorelii*. *Planta medica, 64*(03), 237-241.

Long, B., Niemiera, A. X., Cheng, Z. Y. & Long, C. L. (2010). In vitro propagation of four threatened *Paphiopedilum species* (Orchidaceae). *Plant Cell, Tissue and Organ Culture, 101*(2), 151-162.

Luo, B., Liu, Y., Liu, B., Liu, S., Zhang, B., Zhang, L., . . . Guo, Z. (2018). Yao herbal medicinal market during the Dragon Boat Festival in Jianghua County, China. *Journal of ethnobiology and ethnomedicine, 14*(1), 61.

Lyam, P. T., Musa, M. L., Jamaleddine, Z. O., Okere, U. A. & Odofin, W. T. (2012). The Potential of Temporary Immersion Bioreactors (TIBs) in Meeting Crop Production Demand in Nigeria. *Journal of Biology and Life Science, 3*(1). doi:10.5296/jbls.v3i1.1156

Majumdar, S., Basu, A., Paul, P., Halder, M. & Jha, S. (2013). Bacosides and neuroprotection. *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*, 3639-3660.

Malik, M., Warchoł, M., Kwaśniewska, E. & Pawłowska, B. (2017). Biochemical and morphometric analysis of *Rosa tomentosa* and *Rosa rubiginosa* during application of liquid culture systems for in vitro shoot production. *The Journal of Horticultural Science and Biotechnology, 92*(6), 606-613.

Malik, S., Hossein Mirjalili, M., Fett-Neto, A. G., Mazzafera, P. & Bonfill, M. (2013). Living between two worlds: two-phase culture systems for producing plant secondary metabolites. *Critical Reviews in Biotechnology, 33*(1), 1-22.

Mallón, R., Covelo, P. & Vieitez, A. M. (2011). Improving secondary embryogenesis in *Quercus robur*: application of temporary immersion for mass propagation. *Trees, 26*(3), 731-741. doi:10.1007/s00468-011-0639-6

Malmgren, S. (1996). *Orchid propagation: theory and practice.* Paper presented at the North American native orchids: propagation and production. North American Native Terrestrial Orchid Conference, Germantown, Maryland.

Mamedov, N. & Craker, L. E. (2001). Medicinal plants used for the treatment of bronchial asthma in Russia and Central Asia. *Journal of Herbs, Spices & Medicinal Plants, 8*(2-3), 91-117.

Martre, P., Lacan, D., Just, D. & Teisson, C. (2001). Physiological effects of temporary immersion on *Hevea brasiliensis* callus. *Plant Cell, Tissue and Organ Culture, 67*(1), 25-35.

Masnoddin, M., Repin, R. & Aziz, Z. A. (2016). Micropropagation of an endangered Borneo Orchid, *Paphiopedilum rothschildianum* Callus using Temporary

Immersion Bioreactor System. *Thai Agricultural Research Journal*, *34*(2), 161-171.

McAlister, B., Finnie, J., Watt, M. P. & Blakeway, F. (2005). Use of the temporary immersion bioreactor system (RITA) for production of commercial *Eucalyptus* clones in Mondi Forests (SA). *Plant Cell, Tissue and Organ Culture*, *81*(3), 347-358. doi:10.1007/s11240-004-6658-x

Mead, J. & Bulard, C. (1975). Effects of vitamins and nitrogen sources on asymbiotic germination and development of *Orchis laxiflora* and *Ophrys sphegodes*. *New Phytologist*, *74*(1), 33-40.

Mehrotra, S., Goel, M. K., Kukreja, A. K. & Mishra, B. N. (2007). Efficiency of liquid culture systems over conventional micropropagation: A progress towards commercialization. *African Journal of Biotechnology*, *6*(13).

Mendonça, E. G., Stein, V. C., Carvalho, H. H. d., Santos, B. R., Beijo, L. A. & Paiva, L. V. (2016). The use of continuous, temporary immersion bioreactor system and semisolid culture medium for the production of *Eucalyptus camaldulensis* clones. *Ciência Florestal*, *26*(4), 1211-1224.

Miclea, I. & Zahan, M. (2017). Propagation of *Drosera rotundifolia* and *Drosera capensis* in an in vitro culture system. *Bulletin UASVM Animal Science and Biotechnologies*, *74*, 2.

Mišić, D., Šiler, B., Skorić, M., Djurickovic, M. S., Nestorović Živković, J., Jovanović, V. & Giba, Z. (2013). Secoiridoid glycosides production by *Centaurium maritimum* (L.) Fritch hairy root cultures in temporary immersion bioreactor. *Process Biochemistry*, *48*(10), 1587-1591. doi:10.1016/j.procbio.2013.07.015

Mohanta, Y. K. & Sahoo, S. (2014). In vitro culture of highly valuable medicinal plant *Bacopa monnieri* (L.) Penn. for rapid and mass multiplication. *International Journal of Pharmaceutical Science Invention*, *3*(1), 41-45.

Moreira, A. L., Silva, A. B. d., Santos, A., Reis, C. O. d. & Landgraf, P. R. C. (2013). *Cattleya walkeriana* growth in different micropropagation systems. *Ciência Rural*, *43*(10), 1804-1810.

Mosqueda Frómeta, O., Escalona Morgado, M. M., Teixeira da Silva, J. A., Pina Morgado, D. T. & Daquinta Gradaille, M. A. (2017). In vitro propagation of

Gerbera jamesonii Bolus ex Hooker f. in a temporary immersion bioreactor. *Plant Cell, Tissue and Organ Culture, 129*(3), 543-551. doi:10.1007/s11240-017-1186-7

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497.

Murch, S. J., Liu, C., Romero, R. M. & Saxena, P. K. (2004). In vitro culture and temporary immersion bioreactor production of *Crescentia cujete*. *Plant Cell, Tissue and Organ Culture, 78*(1), 63-68.

Muthiah, J. V. L., Shunmugiah, K. P. & Manikandan, R. (2013). Genetic fidelity assessment of encapsulated in vitro tissues of *Bacopa monnieri* after 6 months of storage by using ISSR and RAPD markers. *Turkish Journal of Botany, 37*(6).

Muthukrishnan, S., Kumar, T. S. & Rao, M. (2013). Effects of Different Media and Organic Additives on Seed Germination of *Geodorum densiflorum* (Lam) Schltr.–An Endangered Orchid. *Indian Journal Social Research, 2*, 23-26.

Nair, S. V., Baranwal, G., Chatterjee, M., Sachu, A., Vasudevan, A. K., Bose, C., . . . Biswas, R. (2016). Antimicrobial activity of plumbagin, a naturally occurring naphthoquinone from *Plumbago rosea*, against *Staphylococcus aureus* and *Candida albicans*. *International Journal of Medical Microbiology, 306*(4), 237-248.

Nanekar, V., Shriram, V., Kumar, V. & Kishor, P. K. (2014). Asymbiotic in vitro seed germination and seedling development of *Eulophia nuda* Lindl., an endangered medicinal orchid. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 84*(3), 837-846.

Niu, G. & Kozai, T. (1997). Simulation of the growth of potato plantlets cultured photoautotrophically in vitro. *Transactions of the ASAE, 40*(1), 255-260.

Paek, K. Y., Hahn, E. J. & Son, S. H. (2001). Application of bioreactors for large-scale micropropagation systems of plants. *In Vitro Cellular and Developmental Biology-Plant, 37*(2), 149-157.

Paek, K. Y., Chakrabarty, D. & Hahn, E. J. (2005). Application of bioreactor systems for large scale production of horticultural and medicinal plants. *Plant Cell, Tissue and Organ Culture, 81*(3), 287-300. doi:10.1007/s11240-004-6648-z

Pakum, W., Watthana, S., Srimuang, K.O. & Kongbangkerd, A. (2016). Influence of Medium Component on In vitro Propagation of Thai's Endangered Orchid: *Bulbophyllum nipondhii* Seidenf. *Plant Tissue Culture and Biotechnology, 26*(1), 37-46.

Parthibhan, S., Benjamin, J. F., Muthukumar, M., Sherif, N. A., Kumar, T. S. & Rao, M. (2012). Influence of nutritional media and photoperiods on in vitro asymbiotic seed germination and seedling development of *Dendrobium aqueum* Lindley. *African Journal of Plant Science, 6*(14), 383-393.

Pedersen, H. Æ., Watthana, S. & Srimuang, K.-O. (2013). Orchids in the torrent: on the circumscription, conservation and rheophytic habit of *Epipactis flava*. *Botanical journal of the Linnean Society, 172*(3), 358-370.

Pérez-Alonso, N., Jiménez, E., De Feria, M., Capote, A., Barbón, R., Quiala, E. & Chávez, M. (2007). Effect of inoculum density and immersion time on the production of potato microtubers in temporary immersion systems and field studies. *Biotecnol Vegetal, 7*, 149-154.

Pérez-Alonso, N., Wilken, D., Gerth, A., Jähn, A., Nitzsche, H.-M., Kerns, G., . . . Jiménez, E. (2009). Cardiotonic glycosides from biomass of *Digitalis purpurea* L. cultured in temporary immersion systems. *Plant Cell, Tissue and Organ Culture, 99*(2), 151-156. doi:10.1007/s11240-009-9587-x

Pérez, M., Bueno, M. A., Escalona, M., Toorop, P., Rodríguez, R. & Cañal, M. J. (2013). Temporary immersion systems (RITA®) for the improvement of cork oak somatic embryogenic culture proliferation and somatic embryo production. *Trees, 27*(5), 1277-1284. doi:10.1007/s00468-013-0876-y

Phrompittayarat, W., Wittaya-areekul, S., Jetiyanon, K., Putalun, W., Tanaka, H. & Ingkaninan, K. (2008). Stability studies of saponins in *Bacopa monnieri* dried ethanolic extracts. *Planta Medica, 74*(14), 1756-1763. doi:10.1055/s-0028-1088311

Piao, X. C., Chakrabarty, D., Hahn, E. J. & Paek, K. Y. (2003). A simple method for mass production of potato microtubers using a bioreactor system. *Current Science*, 1129-1132.

Pitt, J. & Leung, Y. (2016). Cognitive Effects of Nutraceuticals. 29-48.

doi:10.1016/b978-0-12-802147-7.00003-6

Prashanth Kumar, G. & Shiddamallayya, N. (2016). Survey of wild medicinal plants of Hassan district, Karnataka. *Journal of Medicinal Plants, 4*(1), 91-102.

Preil, W. (2005). General introduction: a personal reflection on the use of liquid media for in vitro culture *Liquid culture systems for in vitro plant propagation* (pp. 1-18): Springer.

Putalun, W., Udomsin, O., Yusakul, G., Juengwatanatrakul, T., Sakamoto, S. & Tanaka, H. (2010). Enhanced plumbagin production from in vitro cultures of *Drosera burmanii* using elicitation. *Biotechnology Letters, 32*(5), 721-724. doi:10.1007/s10529-010-0202-3

Quiala, E., Barbón, R., Capote, A., Pérez-Alonso, N., Chávez, M. & Feria, M. (2014). Scaling-up the biomass production of *Cymbopogon citratus* L. in temporary immersion system. *Biotecnol Vegetal, 14*, 67-71.

Quiala, E., Barbón, R., Jimenez, E., Feria, M., Chávez, M., Capote, A. & Pérez, N. (2006). Biomass production of *Cymbopogon citratus* (D.C.) stapf., a medicinal plant, in temporary immersion systems. *In Vitro Cellular and Developmental Biology - Plant, 42*(3), 298-300. doi:10.1079/ivp2006765

Rahman, M. Z., Islam, S. M. S., Chowdhury, A. N. & Subramaniam, S. (2015). Efficient microtuber production of potato in modified nutrient spray bioreactor system. *Scientia Horticulturae, 192*, 369-374. doi:10.1016/j.scienta.2015.06.014

Ramírez-Mosqueda, M. A. & Iglesias-Andreu, L. G. (2016). Evaluation of different temporary immersion systems (BIT®, BIG, and RITA®) in the micropropagation of *Vanilla planifolia* Jacks. *In Vitro Cellular and Developmental Biology - Plant, 52*(2), 154-160. doi:10.1007/s11627-015-9735-4

Ramos-Castellá, A., Iglesias-Andreu, L. G., Bello-Bello, J. & Lee-Espinosa, H. (2014). Improved propagation of *Vanilla* (*Vanilla planifolia* Jacks. ex Andrews) using a temporary immersion system. *In Vitro Cellular and Developmental Biology - Plant, 50*(5), 576-581. doi:10.1007/s11627-014-9602-8

Rathore, S. & Singh, N. (2013). In vitro conservation of *Bacopa monnieri*–an endangered medicinal plant. *Global Journal of BioScience Biotechnol (GJBB), 2*(2), 187-192.

Rejthar, J., Viehmannova, I., Cepkova, P., Fernndez, E. & Milella, L. (2014). In vitro propagation of *Drosera intermedia* as influenced by cytokinins, pH, sucrose, and nutrient concentration. *Emirates Journal of Food and Agriculture, 26*(6), 558. doi:10.9755/ejfa.v26i6.18022

Rice, B. A. (2010). Reversing the roles of predator and prey. *All Flesh is Grass* (pp. 491-518): Springer.

Roca-Pérez, L., Boluda, R., Gavidia, I. & Pérez-Bermúdez, P. (2004). Seasonal cardenolide production and *Dop5βr* gene expression in natural populations of *Digitalis obscura*. *Phytochemistry, 65*(13), 1869-1878.

Roels, S., Noceda, C., Escalona, M., Sandoval, J., Canal, M., Rodriguez, R. & Debergh, P. (2006). The effect of headspace renewal in a temporary immersion bioreactor on plantain (*Musa* AAB) shoot proliferation and quality. *Plant Cell, Tissue and Organ Culture, 84*(2), 155-163.

Roy, A., Bihari, C. & Singh, J. (2017). A review on in vitro propagation and bacoside production in *Bacopa monnieri*. *Scholars Research Library, 5*(2), 21-26.

Saldanha, C. W., Otoni, C. G., de Azevedo, J. L. F., Dias, L. L. C., do Rêgo, M. M. & Otoni, W. C. (2012). A low-cost alternative membrane system that promotes growth in nodal cultures of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]. *Plant Cell, Tissue and Organ Culture, 110*(3), 413-422. doi:10.1007/s11240-012-0162-5

Sandal, I., Bhattacharya, A. & Ahuja, P. S. (2001). An efficient liquid culture system for tea shoot proliferation. *Plant Cell, Tissue and Organ Culture, 65*(1), 75-80.

Santana-Buzzy, N., Canto-Flick, A., Barahona-Pérez, F., del Carmen Montalvo-Peniche, M., Zapata-Castillo, P. Y., Solís-Ruiz, A., . . . de Lourdes Miranda-Ham, M. (2005). Regeneration of habanero pepper (*Capsicum chinense* Jacq.) via organogenesis. *HortScience, 40*(6), 1829-1831.

Santisuk, T., Chayamarit, K., Pooma, R. & Suddee, S. (2006). Thailand red data: plants. *Office of Natural Resources and Environmental Policy and Planning (ONEP), Thailand*.

Scherer, R. F., Holderbaum, D. F., Garcia, A. C., Silva, D. A. d., Steinmacher, D. A. & Guerra, M. P. (2015). Effects of immersion system and gibberellic acid on the

growth and acclimatization of micropropagated pineapple. *Crop Breeding and Applied Biotechnology, 15*(2), 66-71. doi:10.1590/1984-70332015v15n2a13

Schuiteman, A. (1997). Revision of *Agrostophyllum* section *Appendiculopsis* (Orchidaceae), with notes on the systematics of *Agrostophyllum*. *Orchid Monographs, 8*(1), 1-20.

Schumann, A., Berkov, S., Claus, D., Gerth, A., Bastida, J. & Codina, C. (2012). Production of galanthamine by *Leucojum aestivum* shoots grown in different bioreactor systems. *Applied Biochemistry and Biotechnology, 167*(7), 1907-1920. doi:10.1007/s12010-012-9743-3

Seidenfaden, G. & Pedersen, H. Æ. (2002). Contributions to the orchid flora of Thailand XIV. *Nordic Journal of Botany, 22*(5), 525-534.

Shaik, S., Dewir, Y. H., Singh, N. & Nicholas, A. (2010). Micropropagation and bioreactor studies of the medicinally important plant *Lessertia* (Sutherlandia) *frutescens* L. *South African Journal of Botany, 76*(2), 180-186. doi:10.1016/j.sajb.2009.10.005

Sharma, M., Gupta, R., Khajuria, R. K., Mallubhotla, S. & Ahuja, A. (2015). Bacoside biosynthesis during in vitro shoot multiplication in *Bacopa monnieri* (L.) Wettst. grown in Growtek and air lift bioreactor. *Indian Journal of Biotechnology, 14*, 547-551.

Sharma, M., Khajuria, R. K., & Mallubhotla, S. (2013). Annual variation in Bacoside content of *Bacopa monnieri* (L.) Wettst. plants. *International Journal of Pharma and Bio Sciences, 4*(4), 266-271.

Shekarriz, P., Kafi, M., Deilamy, S. D. & Mirmasoumi, M. (2014). Coconut water and peptone improve seed germination and protocorm like body formation of hybrid *Phalaenopsis*. *Agriculture Science Developments, 3*(10), 317-322.

Shimura, H. & Koda, Y. (2004). Micropropagation of *Cypripedium macranthos* var. *rebunense* through protocorm-like bodies derived from mature seeds. *Plant Cell, Tissue and Organ Culture, 78*(3), 273-276.

Shin, Y.-K., Baque, M. A., Elghamedi, S., Lee, E.-J. & Paek, K.-Y. (2011). Effects of Activated Charcoal, Plant Growth Regulators and Ultrasonic Pre-treatments on In vitro Germination and Protocorm Formation of' *Calanthe* Hybrids. *Australian*

Journal of Crop Science, 5(5), 582.

Silva, J. T. (2013). Orchids: advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. *Floriculture and Ornamental Biotechnology, 7*(1), 1-52.

Silva, M. d. A., Medeiros, E., Lima, G., Willadino, L. & Camara, T. (2015). In vitro propagation in Temporary Immersion System of sugarcane plants variety derived from somatic embryos. *Biotecnología Vegetal, 15*(3), 187-191.

Singh, R., Ramakrishna, R., Bhateria, M. & Bhatta, R. S. (2014). In vitro evaluation of *Bacopa monniera* extract and individual constituents on human recombinant monoamine oxidase enzymes. *Phytotherapy Research, 28*(9), 1419-1422.

Singh, R. & Singh, L. (1980). Studies on the anti-anxiety effect of the medyha rasayana drug Brahmi (*Bacopa monniera* Wettst.). *Research in Ayurveda and Siddha, 1*, 133-148.

Snyman, S. J., Nkwanyana, P. D. & Watt, M. P. (2011). Alleviation of hyperhydricity of sugarcane plantlets produced in RITA® vessels and genotypic and phenotypic characterization of acclimated plants. *South African Journal of Botany, 77*(3), 685-692. doi:10.1016/j.sajb.2011.03.004

Sreedhar, R., Venkatachalam, L., Thimmaraju, R., Bhagyalakshmi, N., Narayan, M. & Ravishankar, G. (2008). Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. *Biologia Plantarum, 52*(2), 355-360.

Sreedhar, R. V., Venkatachalam, L. & Neelwarne, B. (2009). Hyperhydricity-Related Morphologic and Biochemical Changes in Vanilla (*Vanilla planifolia*). *Journal of Plant Growth Regulation, 28*(1), 46-57. doi:10.1007/s00344-008-9073-4

Stefano, R. D. & Santos Silva, T. R. (2001). Conservation status of the carnivorous genus *Drosera* in South America and the Caribbean. *Harvard papers in botany*, 253-260.

Steingroewer, J., Bley, T., Georgiev, V., Ivanov, I., Lenk, F., Marchev, A. & Pavlov, A. (2013). Bioprocessing of differentiated plant in vitro systems. *Engineering in Life Sciences, 13*(1), 26-38. doi:10.1002/elsc.201100226

Stewart, S. L. & Kane, M. E. (2006). Asymbiotic seed germination and in vitro seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida

terrestrial orchid. *Plant Cell, Tissue and Organ Culture, 86*(2), 147-158.

Sundriyal, A., Rawat, D. & Singh, A. (2013). Tissue culture, phytochemical and pharmacological study of *Bacopa monnieri*. *Asian Journal of Biochemical and Pharmaceutical Research, 1*(3), 243-260.

Suzuki, R. M., Moreira, V. C., Pescador, R. & de Melo Ferreira, W. (2012). Asymbiotic seed germination and in vitro seedling development of the threatened orchid *Hoffmannseggella cinnabarina*. *In Vitro Cellular and Developmental Biology-Plant, 48*(5), 500-511.

Takayama, S. & Misawa, M. (1981). Mass propagation of *Begonia* x *Hiemalis* plantlets by shake culture. *Plant and Cell Physiology*, *22*(3), 461-467.

Teisson, C. & Alvard, D. (1999). In vitro production of potato microtubers in liquid medium using temporary immersion. *Potato Research, 42*(3-4), 499-504.

Teisson, C., Alvard, D., Berthouly, B., Cote, F., Escalant, J., Etienne, H. & Lartaud, M. (1996). *Simple apparatus to perform plant tissue culture by temporary immersion.* Paper presented at the International Symposium on Plant Production in Closed Ecosystems 440.

Thompson, D. I., Edwards, T. J. & van Staden, J. (2006). Evaluating asymbiotic seed culture methods and establishing *Disa* (Orchidaceae) germinability in vitro: relationships, requirements and first-time reports. *Plant Growth Regulation, 49*(2-3), 269-284.

Tian, J., Chen, Y., Ma, B., He, J., Tong, J. & Wang, Y. (2014). *Drosera peltata* Smith var. *lunata* (Buch.-Ham.) C. B. Clarke as a feasible source of plumbagin: phytochemical analysis and antifungal activity assay. *World Journal of Microbiology and Biotechnology, 30*(2), 737-745. doi:10.1007/s11274-013-1495-x

Tiwari, V., Tiwari, K. N. & Singh, B. D. (2001). Comparative studies of cytokinins on in vitro propagation of *Bacopa monniera*. *Plant Cell, Tissue and Organ Culture, 66*(1), 9-16.

Topoonyanont, N., Pumisutapon, P., Klayraung, S. & Poonnoy, P. (2017). Temporary immersion bioreactors for large scale *Globba* micropropagation. *Acta HortiCulturae* (1155), 51-58. doi:10.17660/ActaHortic.2017.1155.6

Towler, M. J. & Weathers, P. J. (2015). Variations in key artemisinic and other metabolites throughout plant development in *Artemisia annua* L. for potential therapeutic use. *Industrial Crops and Products, 67*, 185-191. doi:10.1016/j.indcrop.2015.01.007

Tripathi, N., Chouhan, D. S., Saini, N. & Tiwari, S. (2012). Assessment of genetic variations among highly endangered medicinal plant *Bacopa monnieri* (L.) from Central India using RAPD and ISSR analysis. *3 Biotechnology*, *2*(4), 327-336. doi:10.1007/s13205-012-0059-3

Tsai, W.-T. & Chu, C.-Y. (2008). Static liquid culture of *Doritaenopsis* seedlings. *HortScience, 43*(1), 206-210.

Vaasa, A. & Rosenberg, V. (2004). Preservation of the rare terrestrial orchids in vitro. *Acta Universitatis Latviensis, 676*, 243-676.

Vacin, E. F. & Went, F. (1949). Some pH changes in nutrient solutions. *Botanical Gazette, 110*(4), 605-613.

Vaidyanathan, R., Vega, A. L., Song, C., Zhou, Q., Tan, B., Berger, S., . . . Eckhardt, L. L. (2013). The interaction of caveolin 3 protein with the potassium inward rectifier channel Kir2. 1 Physiology and pathology related to long QT syndrome 9 (LQT9). *Journal of Biological Chemistry, 288*(24), 17472-17480.

Van Waes, J. & Debergh, P. (1986). In vitro germination of some Western European orchids. *Physiologia Plantarum, 67*(2), 253-261.

Vermeulen, J. J. & Tsukaya, H. (2011). An assumed rheophytic orchid: *Bulbophyllum rheophyton* n. sp., from Borneo. *Plant Systematics and Evolution, 293*(1-4), 71-73.

Vijayakumar, M., Vijayakumar, R. & Stephen, R. (2010). In vitro propagation of *Bacopa monnieri* L.-a multipurpose medicinal plant. *Indian Journal of Science and Technology, 3*(7), 782-787.

Vives, K., Andújar, I., Lorenzo, J. C., Concepción, O., Hernández, M. & Escalona, M. (2017). Comparison of different in vitro micropropagation methods of *Stevia rebaudiana* B. including temporary immersion bioreactor (BIT®). *Plant Cell, Tissue and Organ Culture, 131*(1), 195-199. doi:10.1007/s11240-017-1258-8

Watt, M. P. (2012). The status of temporary immersion system (TIS) technology for

plant micropropagation. *African Journal of Biotechnology*, *11*(76), 14025-14035.

Wawrosch, C. (2009). An Improved 2-step Liquid Culture System for Efficient In Vitro Shoot Proliferation of Sundew (*Drosera rotundifolia* L.). *Scientia Pharmaceutica*. doi:10.3797/scipharm.0908-03

Wawrosch, C. h., Kongbangkerd, A., Kpf, A. & Kopp, B. (2005). Shoot regeneration from nodules of *Charybdis* sp.: a comparison of semisolid, liquid and temporary immersion culture systems. *Plant Cell, Tissue and Organ Culture, 81*(3), 319-322. doi:10.1007/s11240-004-6660-3

Welander, M., Persson, J., Asp, H. & Zhu, L. H. (2014). Evaluation of a new vessel system based on temporary immersion system for micropropagation. *Scientia Horticulturae, 179*, 227-232. doi:10.1016/j.scienta.2014.09.035

Wilken, D., González, E. J., Hohe, A., Jordan, M., Kosky, R. G., Hirschmann, G. S. & Gerth, A. (2005). Comparison of secondary plant metabolite production in cell suspension, callus culture and temporary immersion system *Liquid culture systems for in vitro plant propagation* (pp. 525-537): Springer.

Wu, J. Y., Baque, M. A. & Paek, K. Y. (2010). Establishment of a Large-Scale Micropropagation System for *Anoectochilus formosanus* in Bioreactors. *Acta Horticulturae, 878*, 165-174.

Yadav, A., Ahmad, J., Chaudhary, A. A. & Ahmad, A. (2012). Development of sequence characterized amplified region (SCAR) marker for the authentication of *Bacopa monnieri* (L.) Wettst. *European Journal of Medicinal Plants*, *2*(3), 186.

Yan, H., Liang, C. & Li, Y. (2010). Improved growth and quality of *Siraitia grosvenorii* plantlets using a temporary immersion system. *Plant Cell, Tissue and Organ Culture, 103*(1), 131-135. doi:10.1007/s11240-010-9752-2

Yang, J. F., Piao, X. C., Sun, D. & Lian, M. L. (2010). Production of protocorm-like bodies with bioreactor and regeneration in vitro of *Oncidium* ‘Sugar Sweet’. *Scientia Horticulturae, 125*(4), 712-717. doi:10.1016/j.scienta.2010.05.003

Yang, S.-H. & Yeh, D.-M. (2008). In vitro leaf anatomy, ex vitro photosynthetic behaviors and growth of *Calathea orbifolia* (Linden) Kennedy plants obtained

from semi-solid medium and temporary immersion systems. *Plant Cell, Tissue and Organ Culture, 93*(2), 201-207. doi:10.1007/s11240-008-9363-3

Yesil-Celiktas, O., Gurel, A. & Vardar-Sukan, F. (2010). Large scale cultivation of plant cell and tissue culture in bioreactors. *Transworld Research Network, 37*(661), 2.

Yong, J. W., Ge, L., Ng, Y. F. & Tan, S. N. (2009). The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. *Molecules, 14*(12), 5144-5164.

Yorifuji, E., Ishikawa, N., Okada, H. & Tsukaya, H. (2015). *Arundina graminifolia* var. *revoluta* (Arethuseae, Orchidaceae) has fern-type rheophyte characteristics in the leaves. *Journal of Plant Research, 128*(2), 239-247.

Young, P. S., Murthy, H. & Yoeup, P. K. (2000). Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. *Plant Cell, Tissue and Organ Culture, 63*(1), 67-72.

Yu, T., Xu, Y.-Y., Zhang, Y.-Y., Li, K.-Y., Shao, Y. & Liu, G. (2018). Plumbagin suppresses the human large cell lung cancer cell lines by inhibiting IL-6/STAT3 signaling in vitro. *International Immunopharmacology, 55*, 290-296.

Zeng, S., Huang, W., Wu, K., Zhang, J., Teixeira da Silva, J. A. & Duan, J. (2016). In vitro propagation of *Paphiopedilum* orchids. *Critical Reviews in Biotechnology, 36*(3), 521-534.

Zeng, S., Wu, K., da Silva, J. A. T., Zhang, J., Chen, Z., Xia, N. & Duan, J. (2012). Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. *Scientia Horticulturae, 138*, 198-209.

Zhang, B., Song, L., Bekele, L. D., Shi, J., Jia, Q., Zhang, B., . . . Chen, J. (2018). Optimizing factors affecting development and propagation of *Bletilla striata* in a temporary immersion bioreactor system. *Scientia Horticulturae, 232*, 121-126. doi:10.1016/j.scienta.2018.01.007

Zhang, Y.-Y., Wu, K.-L., Zhang, J.-X., Deng, R.-F., Duan, J., Da Silva, J. A. T., . . . Zeng, S.-J. (2015). Embryo development in association with asymbiotic seed germination in vitro of *Paphiopedilum armeniacum* SC Chen et FY Liu. *Scientific Reports, 5*, 16356.

Zhang, Y., Lee, Y.-I., Deng, L. & Zhao, S. (2013). Asymbiotic germination of immature seeds and the seedling development of *Cypripedium macranthos* Sw., an endangered lady's slipper orchid. *Scientia Horticulturae, 164*, 130-136.

Zhao, Y., Sun, W., Wang, Y., Saxena, P. K. & Liu, C. Z. (2012). Improved mass multiplication of *Rhodiola crenulata* shoots using temporary immersion bioreactor with forced ventilation. *Applied Biochemistry and Biotechnology, 166*(6), 1480-1490. doi:10.1007/s12010-012-9542-x

Zhu, W.-T., Du, J.-Z., Sun, H.-B., Jiang, S.-Y., Chen, X.-J., Sun, H., . . . Wang, H.-L. (2018). In vitro propagation of an endemic and endangered medicinal plant *Notopterygium incisum*. *Plant Cell, Tissue and Organ Culture, 135*(3), 559-563.

Ziv, M. (1999). Developmental and structural patterns of in vitro plants *Morphogenesis in plant tissue cultures* (pp. 235-253): Springer.

Ziv, M. (2005). Simple bioreactors for mass propagation of plants. *Plant Cell, Tissue and Organ Culture, 81*(3), 277-285. doi:10.1007/s11240-004-6649-y